APPLICATION

FOR

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TITLE: TRANSGENICALLY PRODUCED DECORIN

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TRANSGENICALLY PRODUCED DECORIN

This application claims the benefit of a previously filed Provisional Application No. 60/201,932, filed May 5, 2000, the contents of which is incorporated in its entirety.

Background of the Invention

A growing number of recombinant proteins are being developed for therapeutic, diagnostic, agricultural, veterinary, nutritional and other applications; however, many of these proteins may be difficult or expensive to produce in a functional form in the substantial quantities using conventional methods.

Conventional methods often involve inserting the gene responsible for the production of a particular protein into host cells such as bacteria, yeast, or mammalian cells. The cells are grown in culture medium and the desired protein is recovered from the cells or the culture medium. Traditional bacteria or yeast systems are sometimes unable to produce a complex protein in functional form. While some mammalian cells can reproduce complex proteins, they are often difficult and expensive to grow, and produce only protein in relatively low amounts. In addition, non-secreted proteins are relatively difficult to purify from procaryotic or mammalian cells, as they are often not secreted into the culture medium.

Decorin, also known as PG-II or PG-40, is a small proteoglycan produced by fibroblasts. Its core protein has a molecular weight of about 40,000 daltons. The core has been sequenced (Krusius and Ruoslahti, *Proc. Natl. Acad. Sci. USA*, 83:7683 (1986); which is incorporated herein by reference) and it is known to carry a single glycosaminoglycan chain of the chondroitin sulfate/dermatan sulfate type (E. Ruoslahti, *Ann. Rev. Cell Biol.*, 4:229-255 (1988), which is incorporated herein by reference). Most of the core protein of decorin is characterized by the presence of a leucine-rich repeat (LRR) of about 24 amino acids.

Proteoglycans are proteins that carry one or more glycosaminoglycan chains. The known proteoglycans carry out a wide variety of functions and are found in a variety of cellular locations. Many proteoglycans are components of extracellular matrix, where they participate in the assembly of matrix and effect the attachment of cells to the matrix.

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Decorin has been used to prevent TGF-β-induced cell proliferation and extracellular matrix production. Decorin is therefore useful for reducing or preventing pathologies caused by TGFβ-regulated activity, such as cancer, glomerulonephritis and pathologies characterized by excess matrix. In cancer, for example, decorin can be used to destroy TGFβ-1's growth stimulating activity on the cancer cell. Decorin is also useful for reducing or inhibiting wound contraction, which involves proteins of the extracellular matrix.

Summary of the Invention

In general, the invention features, a transgenically produced preparation of decorin, preferably human decorin.

The transgenically produced decorin is produced in a transgenic organism, i.e., a transgenic plant or animal. Preferred transgenic animals include: mammals; birds; reptiles; and amphibians. Suitable mammals include: ruminants; ungulates; domesticated mammals; and dairy animals. Particularly preferred animals include: goats, sheep, camels, cows, pigs, horses, oxen, rabbits and llamas. Suitable birds include chickens, geese, and turkeys. Where the transgenic protein is secreted into the milk of a transgenic animal, the animal should be able to produce at least 1, and more preferably at least 10, or 100, liters of milk per year.

In preferred embodiments, the transgenically produced decorin preparation, preferably as it is made in the transgenic organism, is less than 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, or 5%, glycosylated (in terms of the number of molecules in a preparation which are glycosylated, or in terms of the total contribution of sugar to the molecular weight in a preparation) as compared to the glycosylation of decorin as it is found or as it is isolated from naturally occurring nontransgenic source, or as it is isolated from recombinantly produced decorin in cell culture. Preferably, transgenically produced decorin lacks a glycosaminoglycan (GAG) chain. In preferred embodiment, the preparation of decorin is a preparation wherein less than 50%, 40%, 30%, 20%, 10%, 5%, 2%, or 1% of the decorin molecules have a GAG chain. In another preferred embodiment, the preparation has a ratio of decorin molecules having a GAG chain to decorin molecules lacking a GAG chain of about: 1:2; 1:3; 2:3; 1:4; 3:4; 1:5, 1:6, 1:7, 1:8, 1:9.

In preferred embodiments the transgenic preparation, preferably as it is made in the transgenic organism, includes glycosylated and non-glycosylated forms, and some or all of the glycosylated forms are removed, e.g., from a body fluid, e.g., milk, e.g., by standard protein separation methods.

5   In preferred embodiments, the transgenically produced decorin is made in a mammary gland of the transgenic mammal, e.g., a ruminant, e.g., a goat.

In preferred embodiments, the transgenically produced decorin is secreted into the milk of the transgenic mammal, e.g., a ruminant, e.g., a goat.

In preferred embodiments, the transgenically produced decorin is made under the 10   control of a mammary gland specific promoter, e.g., a milk specific promoter, e.g., a milk serum protein or casein promoter. The milk specific promoter can is a casein promoter, beta lactoglobulin promoter, whey acid protein promoter, or lactalbumin promoter.

In preferred embodiments, the decorin is made under the control of a bladder, or egg specific promoter and decorin is secreted into the urine or into an egg.

15   In preferred embodiments, the transgenically produced decorin preparation differs in average molecular weight, activity, clearance time, or resistance to proteolytic degradation from non-transgenic forms.

In preferred embodiments, the glycosylation of the transgenically produced decorin preparation differs from decorin as it is found or as it is isolated from recombinantly 20   produced decorin in cell culture.

In preferred embodiments, the transgenically produced decorin is expressed from a transgenic organism and the glycosylation of the transgenically produced decorin preparation differs from the glycosylation of decorin as it is found or as it is isolated from a bacterial cell, a yeast cell, an insect cell, a cultured mammalian cell, e.g., a CHO, COS, or HeLa cell. For 25   example, it is different from a protein made by a cultured mammalian cell which has inserted into it a nucleic acid which encodes or directs the expression of decorin.

In preferred embodiments, the electrophoretic mobility of the decorin preparation, e.g., as determined by SDS-PAGE, is different from the electrophoretic mobility of a naturally occurring human decorin; the electrophoretic mobility of the preparation is different

from the electrophoretic mobility of a recombinantly produced human decorin produced in mammalian cells, e.g., CHO, COS, or HeLa cells, or procaryotic cells, e.g., bacteria, or yeast, or insect cells.

In preferred embodiments, the decorin differs by at least one amino acid residue from a naturally occurring human decorin; the decorin differs by at least one amino acid residue from a recombinantly produced human decorin produced in mammalian cells, e.g., CHO, COS, or HeLa cells, procaryotic cells, e.g., bacteria, or yeast, or insect cells.

In preferred embodiments, the decorin the amino acid sequence is that of mammalian or primate, preferably human, decorin.

In preferred embodiments, the preparation includes at least 1, 10, or 100 milligrams of decorin. In preferred embodiments, the preparation includes at least 1, 10, or 100 grams of decorin.

In preferred embodiments, the preparation includes at least 1, 10, 100, or 500 milligrams per milliliter of decorin.

In another aspect, the invention features, an isolated nucleic acid molecule including a decorin protein-coding sequence operatively linked to a tissue specific promoter, e.g., a mammary gland specific promoter sequence that results in the secretion of the protein in the milk of a transgenic mammal.

In preferred embodiments, the promoter is a milk specific promoter, e.g., a milk serum protein or casein promoter. The milk specific promoter can is a casein promoter, beta lactoglobulin promoter, whey acid protein promoter, or lactalbumin promoter.

In preferred embodiments, the promoter is a bladder, or egg specific promoter and decorin is secreted into the urine or into an egg.

In preferred embodiments, the decorin the amino acid sequence is that of mammalian or primate, preferably human, decorin.

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In another aspect, the invention features, a method of making transgenic decorin or a preparation of transgenic decorin. The method includes:

providing a transgenic organism, i.e., a transgenic animal or plant, which includes a transgene which directs the expression of decorin, preferably human decorin;

allowing the transgene to be expressed; and recovering transgenically produced decorin or a preparation of transgenically produced decorin, from the organism or from a product produced by the organism, e.g., milk, seeds, hair, blood, eggs, or urine.

In preferred embodiments, the method further includes:

inserting a nucleic acid which directs the expression of decorin into a cell and allowing the cell to give rise to a transgenic organism;

Preferred transgenic animals include: mammals; birds; reptiles; and amphibians. Suitable mammals include: ruminants; ungulates; domesticated mammals; and dairy animals. Particularly preferred animals include: goats, sheep, camels, cows, pigs, horses, rabbits and mice. Suitable birds include chickens, geese, and turkeys. Where the transgenic protein is secreted into the milk of a transgenic animal, the animal should be able to produce at least 1, and more preferably at least 10, or 100, liters of milk per year.

In preferred embodiments, the transgenically produced decorin preparation, preferably as it is made in the transgenic organism, is less than 80%, 70%, 60% 50%, 40%, 30%, 20%, 10%, or 5%, glycosylated (in terms of the number of molecules in a preparation which are glycosylated, or in terms of the total contribution of sugar to the molecular weight in a preparation) as compared to the glycosylation of decorin as it is found or as it is isolated from naturally occurring nontransgenic source, or as it is isolated from recombinantly produced decorin in cell culture. Preferably, transgenically produced decorin lacks a glycosaminoglycan (GAG) chain. In preferred embodiment, the preparation of decorin, as it is made in the transgenic organism, is a preparation wherein less than 50%, 40%, 30%, 20%, 10%, 5%, 2%, or 1% of the decorin molecules have a GAG chain. In another preferred embodiment, the preparation, as it is made in the transgenic organism, has a ratio of decorin molecules having a GAG chain to decorin molecules lacking a GAG chain of about: 1:2; 1:3; 2:3; 1:4; 3:4; 1:5, 1:6, 1:7, 1:8, 1:9.

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In preferred embodiments the transgenic preparation, preferably as it is made in the transgenic organism, includes glycosylated and non-glycosylated forms, and some or all of the glycosylated forms are removed, e.g., from a body fluid, e.g., milk, e.g., by standard protein separation methods.

In preferred embodiments, the transgenically produced decorin is made in a mammary gland of the transgenic mammal, e.g., a ruminant, e.g., a goat.

In preferred embodiments, the transgenically produced decorin is secreted into the milk of the transgenic mammal, e.g., a ruminant, e.g., a goat.

In preferred embodiments, the transgenically produced decorin is made under the control of a mammary gland specific promoter, e.g., a milk specific promoter, e.g., a milk serum protein or casein promoter. The milk specific promoter can is a casein promoter, beta lactoglobulin promoter, whey acid protein promoter, or lactalbumin promoter.

In preferred embodiments, the decorin is made under the control of a bladder, or egg specific promoter and decorin is secreted into the urine or into an egg.

In preferred embodiments, the transgenically produced decorin preparation differs in average molecular weight, activity, clearance time, or resistance to proteolytic degradation from non-transgenic forms.

In preferred embodiments, the glycosylation of the transgenically produced decorin preparation differs from decorin as it is found or as it is isolated from recombinantly produced decorin in cell culture.

In preferred embodiments, the transgenically produced decorin is expressed from a transgenic organism and the glycosylation of the transgenically produced decorin preparation differs from the glycosylation of decorin as it is found or as it is isolated from a bacterial cell, a yeast cell, an insect cell, a cultured mammalian cell, e.g., a CHO, COS, or HeLa cell. For example, it is different from a protein made by a cultured mammalian cell which has inserted into it a nucleic acid which encodes or directs the expression of decorin.

In preferred embodiments, the electrophoretic mobility of the decorin preparation, e.g., as determined by SDS-PAGE, is different from the electrophoretic mobility of a naturally occurring human decorin; the electrophoretic mobility of the preparation is different

from the electrophoretic mobility of a recombinantly produced human decorin produced in mammalian cells, e.g., CHO, COS, or HeLa cells, or procaryotic cells, e.g., bacteria, or yeast, or insect cells.

In preferred embodiments, the decorin differs by at least one amino acid residue from a naturally occurring human decorin; the decorin differs by at least one amino acid residue from a recombinantly produced human decorin produced in mammalian cells, e.g., CHO, COS, or HeLa cells, procaryotic cells, e.g., bacteria, or yeast, or insect cells.

In preferred embodiments, the decorin the amino acid sequence is that of from mammalian or primate, preferably human, decorin.

In preferred embodiments, the preparation includes at least 1, 10, or 100 milligrams of decorin. In preferred embodiments, the preparation includes at least 1, 10, or 100 grams of decorin.

In preferred embodiments, the preparation includes at least 1, 10, 100, or 500 milligrams per milliliter of decorin.

In another aspect, the invention features, a method for providing a transgenic preparation which includes exogenous decorin in the milk of a transgenic mammal including:

obtaining milk from a transgenic mammal having introduced into its germline a decorin protein-coding sequence operatively linked to a promoter sequence that result in the expression of the protein-coding sequence in mammary gland epithelial cells, thereby secreting the decorin in the milk of the mammal to provide the preparation.

Suitable mammals include: ruminants; ungulates; domesticated mammals; and dairy animals. Particularly preferred mammals include: goats, sheep, camels, cows, pigs, horses, oxen, and llamas. The transgenic mammal should be able to produce at least 1, and more preferably at least 10, or 100, liters of milk per year.

In preferred embodiments, the transgenically produced decorin preparation, preferably as it is made in the transgenic mammal, is less than 80%, 70%, 60%, 50%, 40%, 30%, 25%, 20%, 15%, 10%, 5%, 2.5%, or 1%, glycosylated (in terms of the number of molecules in a preparation which are glycosylated, or in terms of the total contribution of

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sugar to the molecular weight in a preparation) as compared to the glycosylation of decorin as it is found or as it is isolated from naturally occurring nontransgenic source, or as it is isolated from recombinantly produced decorin in cell culture. Preferably, transgenically produced decorin lacks a glycosaminoglycan (GAG) chain. In preferred embodiment, the preparation of decorin, as it is made in the transgenic organism, is a preparation wherein less than 50%, 40%, 30%, 20%, 10%, 5%, 2%, or 1% of the decorin molecules have a GAG chain. In another preferred embodiment, the preparation, as it is made in the transgenic organism, has a ratio of decorin molecules having a GAG chain to decorin molecules lacking a GAG chain of about: 1:2; 1:3; 2:3; 1:4; 3:4; 1:5, 1:6, 1:7, 1:8, 1:9.

In preferred embodiments the transgenic preparation, preferably as it is made in the transgenic mammal, includes glycosylated and non-glycosylated forms, and some or all of the glycosylated forms are removed from the milk, e.g., by standard protein separation methods.

In preferred embodiments, the transgenically produced decorin is made in a mammary gland of the transgenic mammal, e.g., a ruminant, e.g., a goat.

In preferred embodiments, the transgenically produced decorin is secreted into the milk of the transgenic mammal, e.g., a ruminant, e.g., a goat.

In preferred embodiments, the transgenically produced decorin is made under the control of a mammary gland specific promoter, e.g., a milk specific promoter, e.g., a milk serum protein or casein promoter. The milk specific promoter can is a casein promoter, beta lactoglobulin promoter, whey acid protein promoter, or lactalbumin promoter.

In preferred embodiments, the transgenically produced decorin preparation differs in average molecular weight, activity, clearance time, or resistance to proteolytic degradation from non-transgenic forms.

In preferred embodiments, the glycosylation of the transgenically produced decorin preparation differs from decorin as it is found or as it is isolated from recombinantly produced decorin in cell culture.

In preferred embodiments, the transgenically produced decorin is expressed from a transgenic mammal and the glycosylation of the transgenically produced decorin preparation

differs from the glycosylation of decorin as it is found or as it is isolated from a bacterial cell, a yeast cell, an insect cell, a cultured mammalian cell, e.g., a CHO, COS, or HeLa cell. For example, it is different from a protein made by a cultured mammalian cell which has inserted into it a nucleic acid which encodes or directs the expression of decorin.

In preferred embodiments, the electrophoretic mobility of the decorin preparation, e.g., as determined by SDS-PAGE, is different from the electrophoretic mobility of a naturally occurring human decorin; the electrophoretic mobility of the preparation is different from the electrophoretic mobility of a recombinantly produced human decorin produced in mammalian cells, e.g., CHO, COS, or HeLa cells, or procaryotic cells, e.g., bacteria, or yeast, or insect cells.

In preferred embodiments, the decorin differs by at least one amino acid residue from a naturally occurring human decorin; the decorin differs by at least one amino acid residue from a recombinantly produced human decorin produced in mammalian cells, e.g., CHO, COS, or HeLa cells, procaryotic cells, e.g., bacteria, or yeast, or insect cells.

In preferred embodiments, the decorin the amino acid sequence is that of from mammalian or primate, preferably human, decorin.

In preferred embodiments, the milk includes at least 1, 10, 100, 500, 1,000, or 2,000 milligrams per milliliter, of decorin.

In another aspect, the invention features, a transgenic organism, which expresses a transgenic decorin, preferably human decorin, and from which a transgenic preparation of decorin can be obtained.

The transgenic organism is a transgenic plant or animal. Preferred transgenic animals include: mammals; birds; reptiles; and amphibians. Suitable mammals include: ruminants; ungulates; domesticated mammals; and dairy animals. Particularly preferred animals include: goats, sheep, camels, cows, pigs, horses, rabbits and mice. Suitable birds include chickens, geese, and turkeys. Where the transgenic protein is secreted into the milk of a transgenic animal, the animal should be able to produce at least 1, and more preferably at least 10, or 100, liters of milk per year.

In preferred embodiments, the transgenically produced decorin preparation, preferably as it is made in the transgenic organism, is less than 80%, 70%, 60%, 50%, 40%, 30%, 25%, 20%, 15%, 10%, 5%, 2.5%, or 1%, glycosylated (in terms of the number of molecules in a preparation which are glycosylated, or in terms of the total contribution of sugar to the molecular weight in a preparation) as compared to the glycosylation of decorin as it is found or as it is isolated from naturally occurring nontransgenic source, or as it is isolated from recombinantly produced decorin in cell culture. Preferably, transgenically produced decorin lacks a glycosaminoglycan (GAG) chain. In preferred embodiment, the preparation of decorin, as it is made in the transgenic organism, is a preparation wherein less than 50%, 40%, 30%, 20%, 10%, 5%, 2%, or 1% of the decorin molecules have a GAG chain. In another preferred embodiment, the preparation, as it is made in the transgenic organism, has a ratio of decorin molecules having a GAG chain to decorin molecules lacking a GAG chain of about: 1:2; 1:3; 2:3; 1:4; 3:4; 1:5, 1:6, 1:7, 1:8, 1:9.

In preferred embodiments the transgenic preparation, preferably as it is made in the transgenic organism, includes glycosylated and non-glycosylated forms, and some or all of the glycosylated forms are removed, e.g., from a body fluid, e.g., milk, e.g., by standard protein separation methods.

In preferred embodiments, the transgenically produced decorin is made in a mammary gland of the transgenic mammal, e.g., a ruminant, e.g., a goat.

In preferred embodiments, the transgenically produced decorin is secreted into the milk of the transgenic mammal, e.g., a ruminant, e.g., a goat.

In preferred embodiments, the transgenically produced decorin is made under the control of a mammary gland specific promoter, e.g., a milk specific promoter, e.g., a milk serum protein or casein promoter. The milk specific promoter can is a casein promoter, beta lactoglobulin promoter, whey acid protein promoter, or lactalbumin promoter.

In preferred embodiments, the decorin is made under the control of a bladder, or egg specific promoter and is decorin secreted into the urine or into an egg.

In preferred embodiments, the transgenically produced decorin preparation differs in average molecular weight, activity, clearance time, or resistance to proteolytic degradation from non-transgenic forms.

In preferred embodiments, the glycosylation of the transgenically produced decorin preparation differs from decorin as it is found or as it is isolated from recombinantly produced decorin in cell culture.

In preferred embodiments, the transgenically produced decorin is expressed from a transgenic organism and the glycosylation of the transgenically produced decorin preparation differs from the glycosylation of decorin as it is found or as it is isolated from a bacterial cell, a yeast cell, an insect cell, a cultured mammalian cell, e.g., a CHO, COS, or HeLa cell. For example, it is different from a protein made by a cultured mammalian cell which has inserted into it a nucleic acid which encodes or directs the expression of decorin.

In preferred embodiments, the electrophoretic mobility of the decorin preparation, e.g., as determined by SDS-PAGE, is different from the electrophoretic mobility of a naturally occurring human decorin; the electrophoretic mobility of the preparation is different from the electrophoretic mobility of a recombinantly produced human decorin produced in mammalian cells, e.g., CHO, COS, or HeLa cells, or prokaryotic cells, e.g., bacteria, or yeast, or insect cells.

In preferred embodiments, the decorin differs by at least one amino acid residue from a naturally occurring human decorin; the decorin differs by at least one amino acid residue from a recombinantly produced human decorin produced in mammalian cells, e.g., CHO, COS, or HeLa cells, procaryotic cells, e.g., bacteria, or yeast, or insect cells.

In preferred embodiments, the decorin the amino acid sequence is that of from mammalian or primate, preferably human, decorin.

In preferred embodiments, the preparation includes at least 1, 10, or 100 milligrams of decorin. In preferred embodiments, the preparation includes at least 1, 10, or 100 grams of decorin.

In preferred embodiments, the preparation includes at least 1, 10, 100, or 500 milligrams per milliliter of decorin.

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In another aspect, the invention features, a pharmaceutical composition including a therapeutically effective amount of transgenic decorin, or a transgenic preparation of decorin, and a pharmaceutically acceptable carrier.

The transgenic decorin or decorin preparation can be made, e.g., by any method or organism described herein.

The transgenic decorin or decorin preparation can be, e.g., any described herein.

In another aspect, the invention features, a formulation, which includes a transgenically produced decorin preparation, preferably human decorin, and at least one other component, e.g. a nutritional component, other than decorin.

In preferred embodiments, the formulation is a solid or liquid.

In preferred embodiments, the formulation further includes a liquid carrier.

In preferred embodiments, the nutritional component is: a protein, e.g., a milk protein; a vitamin, e.g., vitamin A, vitamin B, vitamin D; a carbohydrate; a mineral, e.g., calcium, phosphorous, iron.

The transgenic decorin or decorin preparation can be made, e.g., by any method or organism described herein.

The transgenic decorin or decorin preparation can be, e.g., any described herein.

In another aspect, the invention features, a nutraceutical, which includes transgenically produced decorin or transgenic preparation of decorin, preferably human decorin, and at least one nutritional component other than decorin.

The transgenic decorin or decorin preparation can be made, e.g., by any method or organism described herein.

The transgenic decorin or decorin preparation can be, e.g., any described herein.

In another aspect, the invention features, a method of providing decorin to a subject in need of decorin. The method includes: administering transgenically produced decorin or a transgenic preparation of decorin to the subject.

In preferred embodiments the subject is: a person, e.g., a patient, in need of decorin. For example, the invention relates to a method for the prevention or reduction of scarring by administering transgenic decorin to a wound. Dermal scarring is a process following a variety of dermal injuries that results in the excessive accumulation of fibrous tissue comprising collagen, fibronectin, and proteoglycans. The induction of fibrous matrix accumulation is a result of growth factor release at the wound site by platelets and inflammatory cells. The principal growth factor believed to induce the deposition of fibrous scar tissue is transforming growth factor-β (TGF-β). Decorin binds and neutralizes a variety of biological functions of TGF-β including the induction of extracellular matrix. Due to the lack of elastic property of this fibrous extracellular matrix, the scar tissue resulting from a severe dermal injury often impairs essential tissue function and can result in an unsightly scar.

The advantage of using transgenic decorin in the methods of the present invention is that it is a normal human protein and is believed to be involved in the natural TGF-β regulatory pathway. Thus, transgenic decorin can be used to prevent or reduce dermal scarring resulting from burn injuries, other invasive skin injuries, and cosmetic or reconstructive surgery.

Decorin-treated wounds have been found to exhibit essentially no detectable scarring compared to control wounds not treated with decorin. The TGF-β-induced scarring process has been shown to be unique to adults and third trimester human fetuses, but is essentially absent in fetuses during the first two trimesters. The absence of scarring in fetal wounds has been correlated with the absence of TGF-β in the wound bed. In contrast, the wound bed of adult tissue is heavily deposited with TGF-β and the fully healed wound is replaced by a reddened, furrowed scar containing extensively fibrous, collagenous matrix. The decorin-treated wounds were histologically normal and resembled fetal wounds in the first two trimesters.

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In another aspect, the invention features a pharmaceutical composition containing transgenic decorin and a pharmaceutically acceptable carrier useful in the above methods. Pharmaceutically acceptable carriers include for example, hyaluronic acid, and aqueous solutions such as bicarbonate-buffers, phosphate buffers, Ringer's solution, and physiological saline supplemented with 5% dextrose or human serum albumin, if desired. The pharmaceutical compositions can also include other agents that promote wound healing known to those skilled in the art. Such agents can include, for example, biologically active chemicals and polypeptides, including RGD-containing polypeptides attached to a biodegradable polymer as described in PCT WO 90/06767, published in Jun. 28, 1990, and incorporated herein by reference. Such polypeptides can be attached to polymers by any means known in the art, including covalent or ionic binding, for example.

In another aspect, the invention features, a method of reducing or inhibiting wound contraction in a subject comprising administering to the subject a pharmaceutical composition comprising transgenic decorin. The invention provides, for example, a method of reducing or inhibiting wound contraction comprising the administration of a pharmaceutical composition comprising transgenic decorin.

In another aspect, the invention features, a method for treating cancer e.g., breast cancer, in a subject comprising administering to a subject a therapeutically effective amount of transgenic decorin.

The transgenic decorin or decorin preparation can be made, e.g., by any method or organism described herein.

The transgenic decorin or decorin preparation can be, e.g., any described herein.

In any of the compositions and methods described herein, the transgenic decorin preparation or formulations can be free of a glycosaminoglycan (GAG) chain resulting in a very homogenous decorin preparation. In another preferred embodiment, the transgenic preparation or formulation is a preparation or formulation wherein less than 50%, 40%, 30%, 20%, 10%, 5%, 2%, or 1% of the decorin molecules have a GAG chain. In another preferred embodiment, the transgenic decorin preparation or formulation has a ratio of decorin

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molecules having a GAG chain to decorin molecules lacking a GAG chain of about: 1:2; 1:3; 2:3; 1:4; 3:4; 1:5, 1:6, 1:7, 1:8, 1:9.

The expression of some transgenic proteins may result in an unwanted effect on the metabolism or health of the transgenic animal or its offspring.

Thus, in another aspect, the invention features a method of producing a transgenic protein in a transgenic animal (wherein the transgenic protein is one which exerts an effect on the metabolism of the transgenic animal) which includes:

expressing the transgenic protein, e.g., in the milk of the transgenic animal; and

treating the transgenic animal to inhibit the effect of the transgenic protein on the transgenic animal.

For example, the animal can be administered, or the animal can transgenically express, a substance which inhibits the effect of the transgenic decorin on the animal, e.g., a substance inhibits an activity of the transgenic decorin. In preferred embodiments, the substance is a polypeptide. The substance can be, by way of example, an enzyme or a receptor, or a fragment thereof, or other molecule which interacts with or binds transgenic decorin. It can act by competitive or non competitive inhibition of an activity of the transgenic decorin, by altering the distribution or transport of the decorin.

If the transgenic protein is found in a particular site in the transgenic animal, e.g., in a tissue, fluid, or organ, the substance can be administered to, or expressed at, that site. E.g., in the case of a transgenic protein which is expressed in the milk of a transgenic animal, the substance can be administered to, or expressed in, the milk of the transgenic animal.

In cases where the substance is transgenically expressed, the transgenic decorin and the substance can be expressed from promoters of the same type, e.g., they can both be expressed from mammary specific promoters, e.g., milk specific promoters. The transgenic decorin and the substance can be expressed from a promoter that results in equal expression of the two, or the two can be expressed from different promoters of different strength. This can result in greater or lesser expression of one or the other. In some cases it will be desirable for the expression of the substance, e.g., on a molar or weight basis, to exceed the

expression of the transgenic decorin. In other cases the opposite will optimize production of the substance. The substance can be expressed at a site other than the one where the decorin is expressed. E.g., the substance can be administered to or expressed at a site in which the transgenic decorin is unwanted, e.g., one into which the transgenic protein is likely to leak, e.g., blood. In preferred embodiments, the transgenic decorin is expressed in the milk of the transgenic animal and the substance is administered to, or expressed in, the blood of the transgenic animal.

In preferred embodiments, an antibody which binds the transgenic decorin is administered to or expressed in the transgenic animal. The antibody can be, by way of example, a single chain antibody or an intrabody. In preferred embodiments, the transgenic decorin is expressed in milk and the antibody is expressed in the blood.

The substance can be administered to, or expressed as a second transgenic protein in, a transgenic animal. However, before producing a transgenic animal, e.g., a large transgenic animal, such as a transgenic goat, the effectiveness of the substance should be tested. This can be accomplished by administering, e.g., by injection, both the decorin and the substance to an animal, e.g., a goat, and monitoring the effect of the decorin on the metabolism or health of the transgenic animal. If the appropriate effect of the substance is seen, the transgenic animal can then be generated. It may be desirable to construct a transgenic animal which expresses transgenic decorin, and to administer candidate substance to that animal in order to evaluate if substance is useful for creating a double transgenic animal, i.e., one which is transgenic for decorin and for the substance.

Host health can also be optimized by tissue specific expression, e.g., expression in the mammary gland, preferably in the milk.

The structure of transgenic decorin can be modified for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., *ex vivo* shelf life and resistance to proteolytic degradation *in vivo*), or to optimize the health of the animal. Such modified decorin, when designed to retain at least one activity of the natural decorin, are considered functional equivalents of the decorin described in more detail herein. Such modified peptide can be produced, for instance, by amino acid substitution, deletion, or addition.

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In preferred embodiments, transgenic decorin can be expressed as a transgenic fusion protein in which it is fused to a second polypeptide. Expression as a fusion protein can be used to optimize the health of the animal, isolation or recovery of the protein, or modify the *ex vivo* shelf life of the protein.

5   In preferred embodiments, the decorin is expressed as a fusion protein with a second polypeptide sequence which fusion results in a minimization of an unwanted effect of the decorin on the metabolism or health of the transgenic animal. The second polypeptide can be one which alters the activity of the decorin of the fusion, e.g., by interfering with an interaction of the decorin moiety with a second molecule, e.g., a receptor, e.g., a decorin

10   receptor. The second protein can be one which alters the tissue distribution of the fusion protein. E.g., the fusion of the second polypeptide to the decorin moiety can prevent migration or transport of the fusion from the site of expression, e.g., mammary tissue or milk, to another site in the transgenic animal, e.g., the circulatory system or the blood.

In preferred embodiments, the second protein is cleaved from the decorin moiety after

15   the fusion protein is expressed or isolated.

The transgenic decorin can be expressed as a fusion protein with a second polypeptide which optimizes the isolation or recovery of the transgenic decorin. E.g., the second polypeptide can optimize isolation by: conferring a desired solubility property on the fusion protein, e.g., by making it more or less soluble; supplying a moiety which simplifies

20   purification, e.g., by supplying an affinity moiety.

As used herein, the glycosylation of two proteins differ if they differ by one or more of the following parameters:

25   (1) the total molecular weight of sugar residues attached to the protein;
(2) the total number of sugar residues attached to the protein;
(3) the subunit composition of the attached sugar residues;
(4) the number of branch points present in the attached sugars;
(5) the location of branch points in the attached sugars;
30   (7) the number of sites at which sugars are attached to the protein;
(8) the position or positions, in the protein, where sugars are attached;
(9) the number of O-linked glycosylation sites; and

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(10) the number of N-linked glycosylation sites.

Two preparations differ from one another if the proportion of transgenic decorin molecules having a selected characteristic, e.g., one or more of those recited immediately above, differs from the proportion of molecules having that characteristic in the second preparation. For example, each of two preparations can contain glycosylated decorin and decorin lacking GAG chains.

A preparation, as used herein, refers to a plurality of molecules produced by one or more transgenic animals. It can include molecules of differing glycosylation or it can be homogenous in this regard. As used herein, the term "a substantially homogenous preparation of transgenically produced decorin" refers to a preparation of decorin wherein less than 10%, 5%, 2% or 1% of the decorin molecules have a GAG chain.

A purified preparation, substantially pure preparation of a polypeptide, or an isolated polypeptide as used herein, means, in the case of a transgenically produced polypeptide, a polypeptide that has been separated from at least one other protein, lipid, or nucleic acid with which it occurs in the transgenic animal or in a fluid, e.g., milk, or other substance, e.g., an egg, produced by the transgenic animal. The polypeptide is preferably separated from substances, e.g., antibodies or gel matrix, e.g., polyacrylamide, which are used to purify it. The polypeptide is preferably constitutes at least 10, 20, 50 70, 80 or 95% dry weight of the purified preparation. Preferably, the preparation contains: sufficient polypeptide to allow protein sequencing; at least 1, 10, or 100 µg of the polypeptide; at least 1, 10, or 100 mg of the polypeptide.

A substantially pure nucleic acid, is a nucleic acid which is one or both of: not immediately contiguous with either one or both of the sequences, e.g., coding sequences, with which it is immediately contiguous (i.e., one at the 5' end and one at the 3' end) in the naturally-occurring genome of the organism from which the nucleic acid is derived; or which is substantially free of a nucleic acid sequence with which it occurs in the organism from which the nucleic acid is derived. The term includes, for example, a recombinant DNA which is incorporated into a vector, e.g., into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction

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endonuclease treatment) independent of other DNA sequences. Substantially pure DNA also includes a recombinant DNA which is part of a hybrid gene encoding additional decorin sequence.

The terms peptides, proteins, and polypeptides are used interchangeably herein.

5   Homology, or sequence identity, as used herein, refers to the sequence similarity between two polypeptide molecules or between two nucleic acid molecules. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are homologous at that position (i.e., as used herein amino acid or nucleic acid "homology" is equivalent to amino

10   acid or nucleic acid "identity"). The percent homology between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % homology = # of identical positions/total # of positions x 100).

For example, if 6 of 10, of the positions in two sequences are matched or homologous then the two sequences are 60% homologous or have 60% sequence identity. By way of

15   example, the DNA sequences ATTGCC and TATGGC share 50% homology or sequence identity. Generally, a comparison is made when two sequences are aligned to give maximum homology or sequence identity.

The comparison of sequences and determination of percent homology between two sequences can be accomplished using a mathematical algorithim. A preferred, non-limiting

20   example of a mathematical algorithim utilized for the comparison of sequences is the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci.* USA 87:2264-68, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci.* USA 90:5873-77. Such an algorithm is incorporated into the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the

25   NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to ITALY nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to ITALY protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul

30   et al., (1997) *Nucleic Acids Res.* 25(17):3389-3402. When utilizing BLAST and Gapped

BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov. Another preferred, non-limiting example of a mathematical algorithim utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

As used herein, the term transgene means a nucleic acid sequence (encoding, e.g., one or more decorin polypeptides), which is partly or entirely heterologous, i.e., foreign, to the transgenic animal or cell into which it is introduced, or, is homologous to an endogenous gene of the transgenic animal or cell into which it is introduced, but which is designed to be inserted, or is inserted, into the animal's genome in such a way as to alter the genome of the cell into which it is inserted (e.g., it is inserted at a location which differs from that of the natural gene or its insertion results in a knockout). A transgene can include one or more transcriptional regulatory sequences and any other nucleic acid, such as introns, that may be necessary for optimal expression and secretion of the selected nucleic acid encoding decorin, e.g., in a mammary gland, all operably linked to the selected decorin nucleic acid, and may include an enhancer sequence. The decorin sequence can be operatively linked to a tissue specific promoter, e.g., mammary gland specific promoter sequence that results in the secretion of the protein in the milk of a transgenic mammal, a urine specific promoter, or an egg specific promoter.

As used herein, the term "transgenic cell" refers to a cell containing a transgene.

A transgenic organism, as used herein, refers to a transgenic animal or plant.

As used herein, a "transgenic animal" is a non-human animal in which one or more, and preferably essentially all, of the cells of the animal contain a heterologous nucleic acid introduced by way of human intervention, such as by transgenic techniques known in the art. The transgene can be introduced into the cell, directly or indirectly by introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus.

Mammals are defined herein as all animals, excluding humans, that have mammary glands and produce milk.

As used herein, a "dairy animal" refers to a milk producing animal. In preferred embodiments, the dairy animal produce large volumes of milk and have long lactating periods, e.g., cows or goats.

As used herein, the term "plant" refers to either a whole plant, a plant part, a plant cell, or a group of plant cells. The class of plants which can be used in the method of the invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants. It includes plants of a variety of ploidy levels, including polyploid, diploid and haploid.

The term "pharmaceutically acceptable composition" refers to compositions which comprise a therapeutically-effective amount of transgenic decorin, formulated together with one or more pharmaceutically acceptable carrier(s).

As used herein, the term "formulation" refers to a composition in solid, e.g., powder, or liquid form, which includes a transgenic decorin. Formulations can provide therapeutical or nutritional benefits. In preferred embodiments, formulations can include at least one nutritional component other than decorin. These formulations may contain a preservative to prevent the growth of microorganisms.

As used herein, the term "nutraceutical," refers to a food substance or part of a food, which includes a transgenic decorin. Nutraceuticals can provide medical or health benefits, including the prevention, treatment or cure of the disease. The transgenic protein will often be present in the nutraceutical at concentration of at least 1 mg/kg. A nutraceutical can include the milk of a transgenic animal.

As used herein, the term "decorin" refers to a proteoglycan or a fragment or analog thereof which has at least one biological activity of decorin. A polypeptide has decorin biological activity if it has one of the following properties: 1) it interacts with, e.g., binds to, an extracellular matrix component, e.g., fibronectin (e.g., the cellular binding domain and/or heparin binding domain of fibronectin), collagen (e.g., collagen I, II, VI, XIV); 2) it modulates, e.g., inhibits, fibrillogenesis; 3) it interacts with, e.g., binds to thrombospondin; 3) it interacts with, e.g., binds to, epidermal growth factor receptor; 4) it modulates, e.g.,

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activates, epidermal growth factor receptor; 5) it modulates, e.g., promotes or inhibits, a signaling pathway, e.g., it promotes a pathway for inducing a kinase inhibitor, e.g., cyclin dependent kinase inhibitor p21; 6) it interacts with, e.g., binds to a growth factor, e.g., TGF-β; 7) it modulates, e.g., inhibits, cell proliferation; 8) it modulates, e.g., inhibits, cell migration; 9) it modulates cell adhesion; and, 10) it modulates matrix assembly and organization. Preferably, human decorin refers to decorin having the amino acid sequence described in Krusius et al. (1986) *Prot. Natl Acad. Sci USA* 83:7683, or variants thereof. Naturally occurring human decorin has a single GAG chain at a serine residue, e.g., serine residue 4 of the amino acid sequence described in Krusius et al. (1986) *Prot. Natl Acad. Sci USA* 83:7683. In addition, naturally occurring human decorin can include two to three asparginine bound oligosacchrides. See, e.g., Glossl (1984) *J. Biol. Chem* 259:14144-14150.

As used herein, the language "subject" is intended to include human and non-human animals. In preferred embodiments, the subject is a person, e.g., a patient, in need of decorin: E.g., a person suffering from a disorder associated with abberant TGF-β activity, e.g., cancer, diabetic kidney disorder, or invasive skin injuries, e.g., burn injuries or a person that has undergone a cosmetic or reconstructive surgery, or a connective tissue disorder, a disorder associated with bone loss or abnormal bone growth (e.g., osteogenesis imperfecta, osteoarthiritis). The term "non-human animals" of the invention includes all vertebrates, e.g., mammals and non-mammals, such as non-human primates, ruminants, birds, amphibians, reptiles. The application of transgenic technology to the commercial production of recombinant proteins in the milk of transgenic animals offers significant advantages over traditional methods of protein production. These advantages include a reduction in the total amount of required capital expenditures, elimination of the need for capital commitment to build facilities early in the product development life cycle, and lower direct production cost per unit for complex proteins. Of key importance is the likelihood that, for certain complex proteins, transgenic production may represent the only technologically and economically feasible method of commercial production.

As used herein, the term "wound contraction" refers to a step in the process of wound healing, wherein the edges of the wound are brought together in an attempt to close the wound (see, for example, Grinnell, J. Cell Biol. 124:401-404(1994)). As used herein, the term "wound healing" is used in its broadest sense to mean the entire process from the time a

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wound is incurred until the physiologic characteristics associated with wound healing are completed. Wound contraction, for example, is part of the wound healing process. Thus, a composition that reduces or inhibits wound contraction can enhance wound healing. It is recognized that wound healing does not necessarily result in the wounded tissue attaining the same level of organization as was present prior to the time of wounding.

Humans produce both glycosylated decorin and decorin lacking one or more GAG chains. Decorin lacking a GAG chain is biologically active. Transgenic organisms, e.g., animals, are a preferred source of decorin lacking a GAG chain resulting in a more homogenous preparation of decorin.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Detailed Description

Transgenic Mammals

Detailed methods for generating non-human transgenic animals are described herein and in the section entitled "Examples" below.

Such methods can involve introducing DNA constructs into the germ line of a mammal to make a transgenic mammal. For example, one or several copies of the construct may be incorporated into the genome of a mammalian embryo by standard transgenic techniques.

Although bovines and goats are preferred, other non-human mammals can be used. Preferred non-human mammals are ruminants, e.g., cows, sheep, camels or goats. Additional examples of preferred non-human animals include horses, pigs, rabbits, mice and rats. For nuclear transfer techniques, the mammal used as the source of cells, e.g., genetically engineered cell, will depend on the transgenic mammal to be obtained. By way of an

example, the genome from a bovine should be used from nuclear transfer with a bovine oocyte.

Methods for the preparation of a variety of transgenic animals are known in the art. Protocols for producing transgenic goats are known in the art. For example, a transgene can be introduced into the germline of a goat by microinjection as described, for example, in Ebert et al. (1994) *Bio/Technology* 12:699, or nuclear transfer techniques as described, for example, in PCT Application WO 98/30683. A protocol for the production of a transgenic pig can be found in White and Yannoutsos, *Current Topics in Complement Research: 64th Forum in Immunology*, pp. 88-94; US Patent No. 5,523,226; US Patent No. 5,573,933; PCT Application WO93/25071; and PCT Application WO95/04744. A protocol for the production of a transgenic rat can be found in Bader and Ganten, *Clinical and Experimental Pharmacology and Physiology*, Supp. 3:S81-S87, 1996. A protocol for the production of a transgenic cow can be found in U.S. Patent No: 5,741,957, PCT Application WO 98/30683, and *Transgenic Animal Technology, A Handbook*, 1994, ed., Carl A. Pinkert, Academic Press, Inc. A protocol for the production of a transgenic sheep can be found in PCT Publication WO 97/07669, and *Transgenic Animal Technology, A Handbook*, 1994, ed., Carl A. Pinkert, Academic Press, Inc.

Transfected Cell Lines

Genetically engineered cells for production of a transgenic mammal by nuclear transfer can be obtained from a cell line into which a nucleic acid of interest, e.g., a nucleic acid which encodes a protein, has been introduced.

A construct can be introduced into a cell via conventional transformation or transfection techniques. As used herein, the terms "transfection" and "transformation" include a variety of techniques for introducing a transgenic sequence into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextrane-mediated transfection, lipofection, or electroporation. In addition, biological vectors, e.g., viral vectors can be used as described below. Suitable methods for transforming or transfecting host cells can be found in Sambrook et al., *Molecular Cloning: A Laboratory Manuel, 2nd ed., Cold*

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Spring Harbor Laboratory, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), and other suitable laboratory manuals.

Two useful approaches are electroporation and lipofection. Brief examples of each are described below.

The DNA construct can be stably introduced into a donor cell line, e.g., an embryonic cell, e.g., an embryonic somatic cell line, by electroporation using the following protocol: the cells are resuspended in PBS at about 4×10^6 cells/ml. Fifty micorgrams of linearized DNA is added to the 0.5 ml cell suspension, and the suspension is placed in a 0.4 cm electrode gap cuvette (Biorad). Electroporation is performed using a Biorad Gene Pulser electroporator with a 330 volt pulse at 25 mA, 1000 microFarad and infinite resistance. If the DNA construct contains a Neomyocin resistance gene for selection, neomyocin resistant clones are selected following incubation with 350 microgram/ml of G418 (GibcoBRL) for 15 days.

The DNA construct can be stably introduced into a donor cell line by lipofection using a protocol such as the following: about 2×10^5 cells are plated into a 3.5 cmiameter well and transfected with 2 micrograms of linearized DNA using LipfectAMINE™ (GibcoBRL). Forty-eight hours after transfection, the cells are split 1:1000 and 1:5000 and, if the DNA construct contains a neomyosin resistance gene for selection, G418 is added to a final concentration of 0.35 mg/ml. Neomyocin resistant clones are isolated and expanded for cyropreservation as well as nuclear transfer.

Tissue-Specific Expression of Proteins

It is often desirable to express a protein, e.g., a heterologous protein, in a specific tissue or fluid, e.g., the milk, blood or urine, of a transgenic animal. The heterologous protein can be recovered from the tissue or fluid in which it is expressed. For example, it is often desirable to express the heterologous protein in milk. Methods for producing a heterologous protein under the control of a milk specific promoter are described below. In addition, other tissue-specific promoters, as well as, other regulatory elements, e.g., signal sequences and sequence which enhance secretion of non-secreted proteins, are described below.

Milk Specific Promoters

Useful transcriptional promoters are those promoters that are preferentially activated in mammary epithelial cells, including promoters that control the genes encoding milk proteins such as caseins, beta lactoglobulin (Clark et al., (1989) Bio/Technology 7: 487-492), whey acid protein (Gordon et al. (1987) Bio/Technology 5: 1183-1187), and lactalbumin (Soulier et al., (1992) FEBS Letts. 297: 13). Casein promoters may be derived from the alpha, beta, gamma or kappa casein genes of any mammalian species; a preferred promoter is derived from the goat beta casein gene (DiTullio, (1992) Bio/Technology 10:74-77). Milk-specific protein promoter or the promoters that are specifically activated in mammary tissue can be derived from cDNA or genomic sequences. Preferably, they are genomic in origin.

DNA sequence information is available for the mammary gland specific genes listed above, in at least one, and often in several organisms. See, e.g., Richards et al., J. Biol. Chem. 256, 526-532 (1981) (α-lactalbumin rat); Campbell et al., Nucleic Acids Res. 12, 8685-8697 (1984) (rat WAP); Jones et al., J. Biol. Chem. 260, 7042-7050 (1985) (rat β-casein); Yu-Lee & Rosen, J. Biol. Chem. 258, 10794-10804 (1983) (rat γ-casein); Hall, Biochem. J. 242, 735-742 (1987) (α-lactalbumin human); Stewart, Nucleic Acids Res. 12, 389 (1984) (bovine αs1 and κ casein cDNAs); Gorodetsky et al., Gene 66, 87-96 (1988) (bovine β casein); Alexander et al., Eur. J. Biochem. 178, 395-401 (1988) (bovine κ casein); Brignon et al., FEBS Lett. 188, 48-55 (1977) (bovine αS2 casein); Jamieson et al., Gene 61, 85-90 (1987), Ivanov et al., Biol. Chem. Hoppe-Seyler 369, 425-429 (1988), Alexander et al., Nucleic Acids Res. 17, 6739 (1989) (bovine β lactoglobulin); Vilotte et al., Biochimie 69, 609-620 (1987) (bovine α-lactalbumin). The structure and function of the various milk protein genes are reviewed by Mercier & Vilotte, J. Dairy Sci. 76, 3079-3098 (1993) (incorporated by reference in its entirety for all purposes). If additional flanking sequences are useful in optimizing expression of the heterologous protein, such sequences can be cloned using the existing sequences as probes. Mammary-gland specific regulatory sequences from different organisms can be obtained by screening libraries from such organisms using known cognate nucleotide sequences, or antibodies to cognate proteins as probes.

Signal Sequences

Useful signal sequences are milk-specific signal sequences or other signal sequences which result in the secretion of eukaryotic or prokaryotic proteins. Preferably, the signal sequence is selected from milk-specific signal sequences, i.e., it is from a gene which encodes a product secreted into milk. Most preferably, the milk-specific signal sequence is related to the milk-specific promoter used in the construct, which are described below. The size of the signal sequence is not critical. All that is required is that the sequence be of a sufficient size to effect secretion of the desired recombinant protein, e.g., in the mammary tissue. For example, signal sequences from genes coding for caseins, e.g., alpha, beta, gamma or kappa caseins, beta lactoglobulin, whey acid protein, and lactalbumin can be used. A preferred signal sequence is the goat β-casein signal sequence.

Signal sequences from other secreted proteins, e.g., proteins secreted by kidney cells, pancreatic cells or liver cells, can also be used. Preferably, the signal sequence results in the secretion of proteins into, for example, urine or blood.

Other Tissue-Specific Promoters

Other tissue-specific promoters which provide expression in a particular tissue can be used. Tissue specific promoters are promoters which are expressed more strongly in a particular tissue than in others. Tissue specific promoters are often expressed essentially exclusively in the specific tissue. For example, if the altered protein is normally expressed in the liver, a liver-specific promoter can be used. This will be the case when a suppressor tRNA is used to alter serum albumin. In this situation, a transgenic sequence encoding the suppressor tRNA can be under the control of a liver-specific promoter.

Tissue-specific promoters which can be used include: a neural-specific promoter, e.g., nestin, Wnt-1, Pax-1, Engrailed-1, Engrailed-2, Sonic hedgehog; a liver-specific promoter, e.g., albumin, alpha-1 antirypsin; a muscle-specific promoter, e.g., myogenin, actin, MyoD, myosin; an oocyte specific promoter, e.g., ZP1, ZP2, ZP3; a testes-specific promoter, e.g., protamin, fertilin, synaptonemal complex protein-1; a blood-specific promoter, e.g., globulin, GATA-1, porphobilinogen deaminase; a lung-specific promoter, e.g., surfactant protein C; a

skin- or wool-specific promoter, e.g., keratin, elastin; endothelium-specific promoters, e.g., Tie-1, Tie-2; and a bone-specific promoter, e.g., BMP.

In addition, general promoters can be used for expression in several tissues. Examples of general promoters include β-actin, ROSA-21, PGK, FOS, c-myc, Jun-A, and Jun-B.

Insulator Sequences

The DNA constructs used to make a transgenic animal can include at least one insulator sequence. The terms "insulator", "insulator sequence" and "insulator element" are used interchangeably herein. An insulator element is a control element which insulates the transcription of genes placed within its range of action but which does not perturb gene expression, either negatively or positively. Preferably, an insulator sequence is inserted on either side of the DNA sequence to be transcribed. For example, the insulator can be positioned about 200 bp to about 1 kb, 5' from the promoter, and at least about 1 kb to 5 kb from the promoter, at the 3' end of the gene of interest. The distance of the insulator sequence from the promoter and the 3' end of the gene of interest can be determined by those skilled in the art, depending on the relative sizes of the gene of interest, the promoter and the enhancer used in the construct. In addition, more than one insulator sequence can be positioned 5' from the promoter or at the 3' end of the transgene. For example, two or more insulator sequences can be positioned 5' from the promoter. The insulator or insulators at the 3' end of the transgene can be positioned at the 3' end of the gene of interest, or at the 3'end of a 3' regulatory sequence, e.g., a 3' untranslated region (UTR) or a 3' flanking sequence.

A preferred insulator is a DNA segment which encompasses the 5' end of the chicken β-globin locus and corresponds to the chicken 5' constitutive hypersensitive site as described in PCT Publication 94/23046, the contents of which is incorporated herein by reference.

DNA Constructs

A cassette which encodes a heterologous protein can be assembled as a construct which includes a promoter, e.g., a promoter for a specific tissue, e.g., for mammary epithelial

cells, e.g., a casein promoter, e.g., a goat beta casein promoter, a milk-specific signal sequence, e.g., a casein signal sequence, e.g., a β-casein signal sequence, and a DNA encoding the heterologous protein.

5 The construct can also include a 3' untranslated region downstream of the DNA sequence coding for the non-secreted protein. Such regions can stabilize the RNA transcript of the expression system and thus increases the yield of desired protein from the expression system. Among the 3' untranslated regions useful in the constructs for use in the invention are sequences that provide a poly A signal. Such sequences may be derived, e.g., from the SV40 small t antigen, the casein 3' untranslated region or other 3' untranslated sequences well

10 known in the art. In one aspect, the 3' untranslated region is derived from a milk specific protein. The length of the 3' untranslated region is not critical but the stabilizing effect of its poly A transcript appears important in stabilizing the RNA of the expression sequence.

Optionally, the construct can include a 5' untranslated region between the promoter and the DNA sequence encoding the signal sequence. Such untranslated regions can be from

15 the same control region from which promoter is taken or can be from a different gene, e.g., they may be derived from other synthetic, semi-synthetic or natural sources. Again their specific length is not critical, however, they appear to be useful in improving the level of expression.

The construct can also include about 10%, 20%, 30%, or more of the N-terminal

20 coding region of a gene preferentially expressed in mammary epithelial cells. For example, the N-terminal coding region can correspond to the promoter used, e.g., a goat β-casein N-terminal coding region.

The construct can be prepared using methods known in the art. The construct can be prepared as part of a larger plasmid. Such preparation allows the cloning and selection of the

25 correct constructions in an efficient manner. The construct can be located between convenient restriction sites on the plasmid so that they can be easily isolated from the remaining plasmid sequences for incorporation into the desired mammal.

Pharmaceutical Compositions

A transgenically produced polypeptide or preparation of the invention can be incorporated into pharmaceutical compositions useful to attenuate, inhibit, treat or prevent a disease or a disorder, e.g., cancer or invasive skin injuries e.g., burn injuries. The compositions should contain a therapeutic or prophylactic amount of the transgenically produced decorin, in a pharmaceutically-acceptable carrier or in the milk of the transgenic animal.

The pharmaceutical carrier can be any compatible, non-toxic substance suitable to deliver the polypeptides to the patient. Sterile water, alcohol, fats, waxes, and inert solids may be used as the carrier. Pharmaceutically-acceptable adjuvants, buffering agents, dispersing agents, and the like, may also be incorporated into the pharmaceutical compositions. The concentration of the transgenically produced peptide or other active agent in the pharmaceutical composition can vary widely, i.e., from less than about 0.1% by weight, usually being at least about 1% weight to as much as 20% by weight or more.

For oral administration, the active ingredient can be administered in solid dosage forms, such as capsules, tablets, and powders, or in liquid dosage forms, such as elixirs, syrups, and suspensions. Active component(s) can be encapsulated in gelatin capsules together with inactive ingredients and powdered carriers, such as glucose, lactose, sucrose, mannitol, starch, cellulose or cellulose derivatives, magnesium stearate, stearic acid, sodium saccharin, talcum, magnesium carbonate and the like. Examples of additional inactive ingredients that may be added to provide desirable color, taste, stability, buffering capacity, dispersion or other known desirable features are red iron oxide, silica gel, sodium lauryl sulfate, titanium dioxide, edible white ink and the like. Similar diluents can be used to make compressed tablets. Both tablets and capsules can be manufactured as sustained release products to provide for continuous release of medication over a period of hours. Compressed tablets can be sugar coated or film coated to mask any unpleasant taste and protect the tablet from the atmosphere, or enteric-coated for selective disintegration in the gastrointestinal tract. Liquid dosage forms for oral administration can contain coloring and flavoring to increase patient acceptance.

For nasal administration, the polypeptides can be formulated as aerosols. The term "aerosol" includes any gas-borne suspended phase of the compounds of the instant invention

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which is capable of being inhaled into the bronchioles or nasal passages. Specifically, aerosol includes a gas-borne suspension of droplets of the compounds of the instant invention, as may be produced in a metered dose inhaler or nebulizer, or in a mist sprayer. Aerosol also includes a dry powder composition of a compound of the instant invention

5   suspended in air or other carrier gas, which may be delivered by insufflation from an inhaler device, for example. See Ganderton & Jones, *Drug Delivery to the Respiratory Tract*, Ellis Horwood (1987); Gonda (1990) *Critical Reviews in Therapeutic Drug Carrier Systems* 6:273-313; and Raeburn et al. (1992) *J. Pharmacol. Toxicol. Methods* 27:143-159.

    The pharmaceutical compositions of the present invention can be administered

10   intravenously or orally. Intradermal or intramuscular administration is also possible in some circumstances. For therapeutic applications, the pharmaceutical compositions are administered to a subject suffering from a disease or disorders, e.g., cancer or invasive skin injuries, in an amount sufficient to inhibit, prevent, or ameliorate the disease. An amount adequate to accomplish this is defined as a "therapeutically-effective amount or dose".

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Formulations

    A formulation includes transgenically produced decorin. In preferred embodiments, the formulation includes transgenic decorin, and at least one nutritional component other than decorin. Nutritional component can be: a protein, e.g., a milk protein; a vitamin, e.g.,

20   vitamin A, vitamin B, vitamin D; a carbohydrate; a mineral, e.g., calcium, phosphorous, iron. The formulation may be in solid or liquid form. In preferred embodiments, the formulation further includes a liquid carrier, e.g., a diluent, e.g., water.

    In preferred embodiments, these formulations are suitable for oral, topical or intravenous or intramuscular administration to a subject. Formulations are useful for

25   therapeutic and/or nutritional applications.

Nutraceuticals

    A transgenic decorin can be included in a nutraceutical. Preferably, the food is milk or milk product obtained from the transgenic mammal or a plant part obtained from a

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transgenic plant both of which express the transgenic protein of the invention. Examples of other nutraceuticals include but are not limited to desserts, ice creams, puddings, and jellies which incorporate the transgenic protein of the invention, as well as soups and beverages. In addition, the isolated transgenic protein of the invention can be provided in powder or tablet form, with or without other known additives, carriers, fillers and diluents. Nutraceuticals are described in Scott Hegenhart, Food Product Design, Dec. 1993.

Transgenic Plants

The transgenic organisms can be a transgenic plant in which the DNA transgene is inserted into the nuclear or plastidic genome. The plant transformation is known as the art. See, in general, *Methods in Enzymology* Vol. 153 ("Recombinant DNA Part D") 1987, Wu and Grossman Eds., Academic Press and European Patent Application EP 693554.

Foreign nucleic acid is can be mechanically transferred by microinjection directly into plant cells by use of micropipettes. Foreign nucleic acid can be transferred into a plant cell by using polyethylene glycol which forms a precipitation complex with the genetic material that is taken up by the cell (Paszkowski et al. (1984) *EMBO J.* 3:2712-22).

Foreign nucleic acid can be introduced into a plant cell by electroporation (Fromm et al. (1985) *Proc. Natl. Acad. Sci. USA* 82:5824). In this technique, plant protoplasts are electroporated in the presence of plasmids or nucleic acids containing the relevant genetic construct. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and form a plant callus. Selection of the transformed plant cells with the transformed gene can be accomplished using phenotypic markers.

Cauliflower mosaic virus (CaMV) can also be used as a vector for introducing foreign nucleic acid into plant cells (Hohn et al. (1982) "Molecular Biology of Plant Tumors," Academic Press, New York, pp. 549-560; Howell, U.S. Pat. No. 4,407,956). CaMV viral DNA genome is inserted into a parent bacterial plasmid creating a recombinant DNA molecule which can be propagated in bacteria. After cloning, the recombinant plasmid again can be cloned and further modified by introduction of the desired DNA sequence into the

unique restriction site of the linker. The modified viral portion of the recombinant plasmid is then excised from the parent bacterial plasmid, and used to inoculate the plant cells or plants.

Another method of introduction of foreign nucleic acid into plant cells is high velocity ballistic penetration by small particles with the nucleic acid either within the matrix of small beads or particles, or on the surface (Klein et al. (1987) *Nature* 327:70-73). Although typically only a single introduction of a new nucleic acid segment is required, this method particularly provides for multiple introductions.

A preferred method of introducing the nucleic acids into plant cells is to infect a plant cell, an explant, a meristem or a seed with *Agrobacterium tumefaciens* transformed with the nucleic acid. Under appropriate conditions known in the art, the transformed plant cells are grown to form shoots, roots, and develop further into plants. The nucleic acids can be introduced into appropriate plant cells, for example, by means of the Ti plasmid of *Agrobacterium tumefaciens*. The Ti plasmid is transmitted to plant cells upon infection by *Agrobacterium tumefaciens*, and is stably integrated into the plant genome (Horsch et al. (1984) "Inheritance of Functional Foreign Genes in Plants," *Science* 233:496-498; Fraley et al. (1983) *Proc. Natl. Acad. Sci. USA* 80:4803).

Ti plasmids contain two regions essential for the production of transformed cells. One of these, named transfer DNA (T DNA), induces tumor formation. The other, termed virulent region, is essential for the introduction of the T DNA into plants. The transfer DNA region, which transfers to the plant genome, can be increased in size by the insertion of the foreign nucleic acid sequence without affecting its transferring ability. By removing the tumor-causing genes so that they no longer interfere, the modified Ti plasmid can then be used as a vector for the transfer of the gene constructs of the invention into an appropriate plant cell.

There are presently at least three different ways to transform plant cells with *Agrobacterium*: (1) co-cultivation of *Agrobacterium* with cultured isolated protoplasts; (2) transformation of cells or tissues with *Agrobacterium*; or (3) transformation of seeds, apices or meristems with *Agrobacterium*. The first method requires an established culture system that allows culturing protoplasts and plant regeneration from cultured protoplasts. The second method requires that the plant cells or tissues can be transformed by *Agrobacterium*

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and that the transformed cells or tissues can be induced to regenerate into whole plants. The third method requires micropropagation.

In the binary system, to have infection, two plasmids are needed: a T-DNA containing plasmid and a vir plasmid. Any one of a number of T-DNA containing plasmids can be used, the only requirement is that one be able to select independently for each of the two plasmids.

After transformation of the plant cell or plant, those plant cells or plants transformed by the Ti plasmid so that the desired DNA segment is integrated can be selected by an appropriate phenotypic marker. These phenotypic markers include, but are not limited to, antibiotic resistance, herbicide resistance or visual observation. Other phenotypic markers are known in the art and can be used in this invention.

Plants from which protoplasts can be isolated and cultured to give whole regenerated plants can be transformed so that whole plants are recovered which contain the transferred foreign gene. Some suitable plants include, for example, species from the genera Fragaria, Lotus, Medicago, Onobrychis, Trifolium, Trigonella, Vigna, Citrus, Linum, Geranium, Manihot, Daucus, Arabidopsis, Brassica, Raphanus, Sinapis, Atropa, Capsicum, Hyoscyamus, Lycopersicon, Nicotiana, Solanum, Petunia, Digitalis, Majorana, Ciohorium, Helianthus, Lactuca, Bromus, Asparagus, Antirrhinum, Hererocallis, Nemesia, Pelargonium, Panicum, Pennisetum, Ranunculus, Senecio, Salpiglossis, Cucumis, Browaalia, Glycine, Lolium, Zea, Triticum, Sorghum, and Datura.

Many plants can be regenerated from cultured cells or tissues. The term "regeneration" as used herein, means growing a whole plant from a plant cell, a group of plant cells, a plant part or a plant piece (e.g. from a protoplast, callus, or tissue part) (*Methods in Enzymology* Vol. 153 ("Recombinant DNA Part D") 1987, Wu and Grossman Eds., Academic Press; also *Methods in Enzymology*, Vol. 118; and Klee et al., (1987) *Annual Review of Plant Physiology*, 38:467-486).

Plant regeneration from cultural protoplasts is described in Evans et al., "Protoplasts Isolation and Culture," *Handbook of Plant Cell Cultures* 1:124-176 (MacMillan Publishing Co. New York 1983); M.R. Davey, "Recent Developments in the Culture and Regeneration of Plant Protoplasts," *Protoplasts* (1983)-Lecture Proceedings, pp. 12-29, (Birkhauser, Basal

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1983); P.J. Dale, "Protoplast Culture and Plant Regeneration of Cereals and Other Recalcitrant Crops," *Protoplasts* (1983)-Lecture Proceedings, pp. 31-41, (Birkhauser, Basel 1983); and H. Binding, "Regeneration of Plants," *Plant Protoplasts*, pp. 21-73, (CRC Press, Boca Raton 1985).

Regeneration from protoplasts varies from species to species of plants, but generally a suspension of transformed protoplasts containing copies of the exogenous sequence is first generated. In certain species, embryo formation can then be induced from the protoplast suspension, to the stage of ripening and germination as natural embryos. The culture media can contain various amino acids and hormones, such as auxin and cytokinins. It can also be advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Shoots and roots normally develop simultaneously. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is fully reproducible and repeatable.

In vegetatively propagated crops, the mature transgenic plants are propagated by the taking of cuttings or by tissue culture techniques to produce multiple identical plants for trialling, such as testing for production characteristics. Selection of a desirable transgenic plant is made and new varieties are obtained thereby, and propagated vegetatively for commercial sale. In seed propagated crops, the mature transgenic plants are self crossed to produce a homozygous inbred plant. The inbred plant produces seed containing the gene for the newly introduced foreign gene activity level. These seeds can be grown to produce plants that have the selected phenotype. The inbreds according to this invention can be used to develop new hybrids. In this method a selected inbred line is crossed with another inbred line to produce the hybrid.

Parts obtained from the regenerated plant, such as flowers, seeds, leaves, branches, fruit, and the like are covered by the invention, provided that these parts comprise cells which have been so transformed. Progeny and variants, and mutants of the regenerated plants are also included within the scope of this invention, provided that these parts comprise the introduced DNA sequences. Progeny and variants, and mutants of the regenerated plants are also included within the scope of this invention.

However, any additional attached vector sequences which confers resistance to degradation of the nucleic acid fragment to be introduced, which assists in the process of genomic integration or provides a means to easily select for those cells or plants which are transformed are advantageous and greatly decrease the difficulty of selecting useable

5   transgenic plants or plant cells.

Selection of transgenic plants or plant cells is typically based upon a visual assay, such as observing color changes (e.g., a white flower, variable pigment production, and uniform color pattern on flowers or irregular patterns), but can also involve biochemical assays of either enzyme activity or product quantitation. Transgenic plants or plant cells are

10   grown into plants bearing the plant part of interest and the gene activities are monitored, such as by visual appearance (for flavonoid genes) or biochemical assays (Northern blots); Western blots; enzyme assays and flavonoid compound assays, including spectroscopy, see, Harborne et al. (Eds.), (1975) *The Flavonoids*, Vols. 1 and 2, [Acad. Press]). Appropriate plants are selected and further evaluated. Methods for generation of genetically engineered

15   plants are further described in US Patent No. 5,283,184, US Patent No. 5, 482,852, and European Patent Application EP 693 554.

Purification from milk

The transgenic protein can be produced in milk at relatively high concentrations and

20   in large volumes, providing continuous high level output of normally processed peptide that is easily harvested from a renewable resource. There are several different methods known in the art for isolation of proteins form milk.

Milk proteins usually are isolated by a combination of processes. Raw milk first is fractionated to remove fats, for example, by skimming, centrifugation, sedimentation (H.E.

25   Swaisgood, Developments in Dairy Chemistry, I: Chemistry of Milk Protein, Applied Science Publishers, NY, 1982), acid precipitation (U.S. Patent No. 4,644,056) or enzymatic coagulation with rennin or chymotrypsin (Swaisgood, *ibid.*). Next, the major milk proteins may be fractionated into either a clear solution or a bulk precipitate from which the specific protein of interest may be readily purified.

30   French Patent No. 2487642 describes the isolation of milk proteins from skim milk or whey by membrane ultrafiltration in combination with exclusion chromatography or ion

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exchange chromatography. Whey is first produced by removing the casein by coagulation with rennet or lactic acid. U.S. Patent No. 4,485,040 describes the isolation of an alpha-lactoglobulin-enriched product in the retentate from whey by two sequential ultrafiltration steps. U.S. Patent No. 4,644,056 provides a method for purifying immunoglobulin from milk or colostrum by acid precipitation at pH 4.0-5.5, and sequential cross-flow filtration first on a membrane with 0.1 - 1.2 micrometer pore size to clarify the product pool and then on a membrane with a separation limit of 5 - 80 kd to concentrate it.

Similarly, U.S. Patent No. 4,897,465 teaches the concentration and enrichment of a protein such as immunoglobulin from blood serum, egg yolks or whey by sequential ultrafiltration on metallic oxide membranes with a pH shift. Filtration is carried out first at a pH below the isoelectric point (pI) of the selected protein to remove bulk contaminants from the protein retentate, and next at a pH above the pI of the selected protein to retain impurities and pass the selected protein to the permeate. A different filtration concentration method is taught by European Patent No. EP 467 482 B 1 in which defatted skim milk is reduced to pH 3-4, below the pI of the milk proteins, to solubilize both casein and whey proteins. Three successive rounds of ultrafiltration or diafiltration then concentrate the proteins to form a retentate containing 15-20% solids of which 90% is protein. Alternatively, British Patent Application No. 2179947 discloses the isolation of lactoferrin from whey by ultrafiltration to concentrate the sample, followed by weak cation exchange chromatography at approximately a neutral pH. No measure of purity is reported. In PCT Publication No. WO 95/22258, a protein such as -lactoferrin is recovered from milk that has been adjusted to high ionic strength by the addition of concentrated salt, followed by cation exchange chromatography.

In all of these methods, milk or a fraction thereof is first treated to remove fats, lipids, and other particulate matter that would foul filtration membranes or chromatography media. The initial fractions thus produced may consist of casein, whey, or total milk protein, from which the protein of interest is then isolated.

PCT Patent Publication No. WO 94/19935 discloses a method of isolating a biologically active protein from whole milk by stabilizing the solubility of total milk proteins with a positively charged agent such as arginine, imidazole or Bis-Tris. This treatment forms a clarified solution from which the protein may be isolated, e.g., by filtration through membranes that otherwise would become clogged by precipitated proteins.

USSN 08/648,235 discloses a method for isolating a soluble milk component, such as a peptide, in its biologically active form from whole milk or a milk fraction by tangential flow filtration. Unlike previous isolation methods, this eliminates the need for a first fractionation of whole milk to remove fat and casein micelles, thereby simplifying the process and avoiding losses of recovery and bioactivity. This method may be used in combination with additional purification steps to further remove contaminants and purify the component of interest.

The Sequence of Decorin

As used herein, the term "decorin" refers to a proteoglycan or a fragment or analog thereof which has at least one biological activity of decorin. A polypeptide has decorin biological activity if it has one of the following properties: 1) it interacts with, e.g., binds to, an extracellular matrix component, e.g., fibronectin (e.g., the cellular binding domain and/or heparin binding domain of fibronectin), collagen (e.g., collagen I, II, VI, XIV); 2) it modulates, e.g., inhibits, fibrillogenesis; 3) it interacts with, e.g., binds to thrombospondin; 3) it interacts with, e.g., binds to, epidermal growth factor receptor; 4) it modulates, e.g., activates, epidermal growth factor receptor; 5) it modulates, e.g., promotes or inhibits, a signaling pathway, e.g., it promotes a pathway for inducing a kinase inhibitor, e.g., cyclin dependent kinase inhibitor p21; 6) it interacts with, e.g., binds to a growth factor, e.g., TGF-β; 7) it modulates, e.g., inhibits, cell proliferation; 8) it modulates, e.g., inhibits, cell migration; 9) it modulates cell adhesion; and, 10) it modulates matrix assembly and organization.

The sequence encoding decorin is known. Preferably, human decorin refers to decorin having the amino acid sequence described in Krusius et al. (1986) *Prot. Natl Acad. Sci USA* 83:7683, or variants thereof. Naturally occurring human decorin has a single GAG chain at a serine residue, e.g., serine residue 4 of the amino acid sequence described in Krusius et al. (1986) *Prot. Natl Acad. Sci USA* 83:7683. In addition, naturally occurring human decorin can include two to three asparginine bound oligosacchrides. See, e.g., Glossl (1984) *J. Biol. Chem* 259:14144-14150.

Fragments and Analogs of Decorin

The transgenically produced decorin can have the amino acid sequence of a naturally occurring protein or it can be a fragment or analog of a naturally occurring protein.

In a preferred embodiment, the decorin polypeptide differs in amino acid sequence at up to, but not more than, 1, 2, 3, 5, or 10 residues, from the sequence of naturally occurring decorin. In other preferred embodiments, the decorin polypeptide differs in amino acid sequence at up to, but not more than, 1, 2, 3, 5, or 10 % of the residues from a sequence of naturally occurring decorin. In preferred embodiments, the differences are such that the decorin polypeptide exhibits an decorin biological activity. In other preferred embodiments, the differences are such that the decorin polypeptide does not have decorin biological activity. In preferred embodiments, one or more, or all of the differences are conservative amino acid changes. In other preferred embodiments, one or more, or all of the differences are other than conservative amino acid changes.

In preferred embodiments, the decorin polypeptide is a fragment of a full length decorin polypeptide, e.g., a fragment of a naturally occurring decorin polypeptide.

In preferred embodiments: the fragment is at least 5, 10, 20, 50, 100, or 150 amino acids in length; the fragment is equal to or less than 200, 150, 100, 50 amino acid residues in length; the fragment has a biological activity of a naturally occurring decorin; the fragment is either, an agonist or an antagonist, of a biological activity of a naturally occurring decorin; the fragment can inhibit, e.g., competitively or non competitively inhibit, the binding of decorin to a receptor, or an enzyme.

In preferred embodiments, the fragment it has at least 60, and more preferably at least 70, 80, 90, 95, 99, or 100 % sequence identity with the corresponding amino acid sequence of naturally occurring decorin.

In preferred embodiments, the fragment is a fragment of a vertebrate, e.g., a mammalian, e.g. a primate, e.g., a human decorin polypeptide.

In a preferred embodiment, the fragment differs in amino acid sequence at up to, but not more than, 1, 2, 3, 5, or 10 residues, from the corresponding residues of naturally occurring decorin. In other preferred embodiments, the fragment differs in amino acid

sequence at up to 1, 2, 3, 5, or 10 % of the residues from the corresponding residues of naturally occurring decorin. In preferred embodiments, the differences are such that the fragment exhibits a decorin biological activity. In other preferred embodiments, the differences are such that the fragment does not have decorin biological activity. In preferred

5   embodiments, one or more, or all of the differences are conservative amino acid changes. In other preferred embodiments one or more, or all of the differences are other than conservative amino acid changes.

Polypeptides of the invention include those which arise as a result of the existence of multiple genes, alternative transcription events, alternative RNA splicing events, and

10   alternative translational and postranslational events.

Production of Fragments and Analogs

One skilled in the art can alter the disclosed structure of decorin by producing

15   fragments or analogs, and test the newly produced structures for activity. Examples of prior art methods which allow the production and testing of fragments and analogs are discussed below. These, or other methods, can be used to make and screen fragments and analogs of a decorin polypeptide. In preferred embodiments, the decorin structure modified is human decorin.

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Generation of Fragments

Fragments of a protein can be produced in several ways, e.g., recombinantly, by proteolytic digestion, or by chemical synthesis. Internal or terminal fragments of a polypeptide can be generated by removing one or more nucleotides from one end (for a

25   terminal fragment) or both ends (for an internal fragment) of a nucleic acid which encodes the polypeptide. Expression of the mutagenized DNA produces polypeptide fragments. Digestion with "end-nibbling" endonucleases can thus generate DNA's which encode an array of fragments. DNA's which encode fragments of a protein can also be generated by random shearing, restriction digestion or a combination of the above-discussed methods.

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Fragments can also be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, peptides of the present invention may be arbitrarily divided into fragments of desired length with no overlap of the fragments, or divided into overlapping fragments of a desired length.

5

Generation of Analogs: Production of Altered DNA and Peptide Sequences by Random Methods

Amino acid sequence variants of a protein can be prepared by random mutagenesis of DNA which encodes a protein or a particular domain or region of a protein. Useful methods include PCR mutagenesis and saturation mutagenesis. A library of random amino acid sequence variants can also be generated by the synthesis of a set of degenerate oligonucleotide sequences. (Methods for screening proteins in a library of variants are elsewhere herein.)

PCR Mutagenesis

In PCR mutagenesis, reduced Taq polymerase fidelity is used to introduce random mutations into a cloned fragment of DNA (Leung et al., 1989, *Technique* 1:11-15). This is a very powerful and relatively rapid method of introducing random mutations. The DNA region to be mutagenized is amplified using the polymerase chain reaction (PCR) under conditions that reduce the fidelity of DNA synthesis by Taq DNA polymerase, e.g., by using a dGTP/dATP ratio of five and adding Mn^{2+} to the PCR reaction. The pool of amplified DNA fragments are inserted into appropriate cloning vectors to provide random mutant libraries.

Saturation Mutagenesis

Saturation mutagenesis allows for the rapid introduction of a large number of single base substitutions into cloned DNA fragments (Mayers et al., 1985, *Science* 229:242). This technique includes generation of mutations, e.g., by chemical treatment or irradiation of single-stranded DNA *in vitro,* and synthesis of a complimentary DNA strand. The mutation

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frequency can be modulated by modulating the severity of the treatment, and essentially all possible base substitutions can be obtained. Because this procedure does not involve a genetic selection for mutant fragments both neutral substitutions, as well as those that alter function, are obtained. The distribution of point mutations is not biased toward conserved sequence elements.

Degenerate Oligonucleotides

A library of homologs can also be generated from a set of degenerate oligonucleotide sequences. Chemical synthesis of a degenerate sequences can be carried out in an automatic DNA synthesizer, and the synthetic genes then ligated into an appropriate expression vector. The synthesis of degenerate oligonucleotides is known in the art (see for example, Narang, SA (1983) *Tetrahedron* 39:3; Itakura et al. (1981) *Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules*, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al. (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al. (1984) *Science* 198:1056; Ike et al. (1983) *Nucleic Acid Res.* 11:477. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al. (1990) *Science* 249:386-390; Roberts et al. (1992) *PNAS* 89:2429-2433; Devlin et al. (1990) *Science* 249: 404-406; Cwirla et al. (1990) *PNAS* 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815).

Generation of Analogs: Production of Altered DNA and Peptide Sequences by Directed Mutagenesis

Non-random or directed, mutagenesis techniques can be used to provide specific sequences or mutations in specific regions. These techniques can be used to create variants which include, e.g., deletions, insertions, or substitutions, of residues of the known amino acid sequence of a protein. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conserved amino acids and then with more radical choices depending upon results achieved, (2) deleting the target residue, or (3) inserting residues of the same or a different class adjacent to the located site, or combinations of options 1-3.

Alanine Scanning Mutagenesis

Alanine scanning mutagenesis is a useful method for identification of certain residues or regions of the desired protein that are preferred locations or domains for mutagenesis, Cunningham and Wells (*Science* 244:1081-1085, 1989). In alanine scanning, a residue or

5 group of target residues are identified (e.g., charged residues such as Arg, Asp, His, Lys, and Glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine). Replacement of an amino acid can affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions are then refined by introducing

10 further or other variants at or for the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to optimize the performance of a mutation at a given site, alanine scanning or random mutagenesis may be conducted at the target codon or region and the expressed desired protein subunit variants are screened for the optimal combination of

15 desired activity.

Oligonucleotide-Mediated Mutagenesis

Oligonucleotide-mediated mutagenesis is a useful method for preparing substitution, deletion, and insertion variants of DNA, see, e.g., Adelman et al., (*DNA* 2:183, 1983).

20 Briefly, the desired DNA is altered by hybridizing an oligonucleotide encoding a mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of the desired protein. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for

25 the selected alteration in the desired protein DNA. Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using

techniques known in the art such as that described by Crea et al. (*Proc. Natl. Acad. Sci.* USA, 75: 5765[1978]).

Cassette Mutagenesis

5   Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells et al. (*Gene*, 34:315[1985]). The starting material is a plasmid (or other vector) which includes the protein subunit DNA to be mutated. The codon(s) in the protein subunit DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites

10   exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the desired protein subunit DNA. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using

15   standard procedures. The two strands are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3' and 5' ends that are comparable with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated desired protein subunit DNA sequence.

20

Combinatorial Mutagenesis

Combinatorial mutagenesis can also be used to generate mutants. E.g., the amino acid sequences for a group of homologs or other related proteins are aligned, preferably to promote the highest homology possible. All of the amino acids which appear at a given

25   position of the aligned sequences can be selected to create a degenerate set of combinatorial sequences. The variegated library of variants is generated by combinatorial mutagenesis at the nucleic acid level, and is encoded by a variegated gene library. For example, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential sequences are expressible as individual peptides, or alternatively,

30   as a set of larger fusion proteins containing the set of degenerate sequences.

<u>Primary High-Through-Put Methods for Screening Libraries of Peptide Fragments or Homologs</u>

Various techniques are known in the art for screening generated mutant gene

5   products. Techniques for screening large gene libraries often include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the genes under conditions in which detection of a desired activity, e.g., in this case, the interaction, e.g., binding of decorin to a decorin-interacting polypeptide, e.g., decorin receptor, or the interaction, e.g., binding of a candidate polypeptide with a

10   decorin polypeptide facilitate relatively easy isolation of the vector encoding the gene whose product was detected. Each of the techniques described below is amenable to high through-put analysis for screening large numbers of sequences created, e.g., by random mutagenesis techniques.

15   <u>Two Hybrid Systems</u>

Two hybrid assays such as the system described above (as with the other screening methods described herein), can be used to identify fragments or analogs of a decorin polypeptide which binds to a decorin interactor. These may include agonists, superagonists, and antagonists.

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<u>Display Libraries</u>

In one approach to screening assays, the candidate peptides are displayed on the surface of a cell or viral particle, and the ability of particular cells or viral particles to bind an appropriate receptor protein via the displayed product is detected in a "panning assay". For

25   example, the gene library can be cloned into the gene for a surface membrane protein of a bacterial cell, and the resulting fusion protein detected by panning (Ladner et al., WO 88/06630; Fuchs et al. (1991) *Bio/Technology* 9:1370-1371; and Goward et al. (1992) *TIBS* 18:136-140). In a similar fashion, a detectably labeled ligand can be used to score for potentially functional peptide homologs. Fluorescently labeled ligands, e.g., receptors, can

be used to detect homolog which retain ligand-binding activity. The use of fluorescently labeled ligands, allows cells to be visually inspected and separated under a fluorescence microscope, or, where the morphology of the cell permits, to be separated by a fluorescence-activated cell sorter.

5   A gene library can be expressed as a fusion protein on the surface of a viral particle. For instance, in the filamentous phage system, foreign peptide sequences can be expressed on the surface of infectious phage, thereby conferring two significant benefits. First, since these phage can be applied to affinity matrices at concentrations well over 10^{13} phage per milliliter, a large number of phage can be screened at one time. Second, since each

10   infectious phage displays a gene product on its surface, if a particular phage is recovered from an affinity matrix in low yield, the phage can be amplified by another round of infection. The group of almost identical *E. coli* filamentous phages M13, fd., and fl are most often used in phage display libraries. Either of the phage gIII or gVIII coat proteins can be used to generate fusion proteins without disrupting the ultimate packaging of the viral

15   particle. Foreign epitopes can be expressed at the NH_2-terminal end of pIII and phage bearing such epitopes recovered from a large excess of phage lacking this epitope (Ladner et al. PCT publication WO 90/02909; Garrard et al., PCT publication WO 92/09690; Marks et al. (1992) *J. Biol. Chem.* 267:16007-16010; Griffiths et al. (1993) *EMBO J* 12:725-734; Clackson et al. (1991) *Nature* 352:624-628; and Barbas et al. (1992) *PNAS* 89:4457-4461).

20   A common approach uses the maltose receptor of *E. coli* (the outer membrane protein, LamB) as a peptide fusion partner (Charbit et al. (1986) *EMBO* 5, 3029-3037). Oligonucleotides have been inserted into plasmids encoding the LamB gene to produce peptides fused into one of the extracellular loops of the protein. These peptides are available for binding to ligands, e.g., to antibodies, and can elicit an immune response when the cells

25   are administered to animals. Other cell surface proteins, e.g., OmpA (Schorr et al. (1991) *Vaccines 91*, pp. 387-392), PhoE (Agterberg, et al. (1990) *Gene* 88, 37-45), and PAL (Fuchs et al. (1991) *Bio/Tech* 9, 1369-1372), as well as large bacterial surface structures have served as vehicles for peptide display. Peptides can be fused to pilin, a protein which polymerizes to form the pilus-a conduit for interbacterial exchange of genetic information (Thiry et al.

30   (1989) *Appl. Environ. Microbiol.* 55, 984-993). Because of its role in interacting with other

cells, the pilus provides a useful support for the presentation of peptides to the extracellular environment. Another large surface structure used for peptide display is the bacterial motive organ, the flagellum. Fusion of peptides to the subunit protein flagellin offers a dense array of may peptides copies on the host cells (Kuwajima et al. (1988) *Bio/Tech.* 6, 1080-1083).

5 Surface proteins of other bacterial species have also served as peptide fusion partners. Examples include the *Staphylococcus* protein A and the outer membrane protease IgA of *Neisseria* (Hansson et al. (1992) *J. Bacteriol.* 174, 4239-4245 and Klauser et al. (1990) *EMBO J.* 9, 1991-1999).

In the filamentous phage systems and the LamB system described above, the physical
10 link between the peptide and its encoding DNA occurs by the containment of the DNA within a particle (cell or phage) that carries the peptide on its surface. Capturing the peptide captures the particle and the DNA within. An alternative scheme uses the DNA-binding protein LacI to form a link between peptide and DNA (Cull *et al.* (1992) *PNAS USA* 89:1865-1869). This system uses a plasmid containing the LacI gene with an oligonucleotide
15 cloning site at its 3'-end. Under the controlled induction by arabinose, a LacI-peptide fusion protein is produced. This fusion retains the natural ability of LacI to bind to a short DNA sequence known as LacO operator (LacO). By installing two copies of LacO on the expression plasmid, the LacI-peptide fusion binds tightly to the plasmid that encoded it. Because the plasmids in each cell contain only a single oligonucleotide sequence and each
20 cell expresses only a single peptide sequence, the peptides become specifically and stably associated with the DNA sequence that directed its synthesis. The cells of the library are gently lysed and the peptide-DNA complexes are exposed to a matrix of immobilized receptor to recover the complexes containing active peptides. The associated plasmid DNA is then reintroduced into cells for amplification and DNA sequencing to determine the
25 identity of the peptide ligands. As a demonstration of the practical utility of the method, a large random library of dodecapeptides was made and selected on a monoclonal antibody raised against the opioid peptide dynorphin B. A cohort of peptides was recovered, all related by a consensus sequence corresponding to a six-residue portion of dynorphin B. (Cull et al. (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89-1869)

30 This scheme, sometimes referred to as peptides-on-plasmids, differs in two important ways from the phage display methods. First, the peptides are attached to the C-terminus of

the fusion protein, resulting in the display of the library members as peptides having free carboxy termini. Both of the filamentous phage coat proteins, pIII and pVIII, are anchored to the phage through their C-termini, and the guest peptides are placed into the outward-extending N-terminal domains. In some designs, the phage-displayed peptides are presented

5   right at the amino terminus of the fusion protein. (Cwirla, et al. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87, 6378-6382) A second difference is the set of biological biases affecting the population of peptides actually present in the libraries. The LacI fusion molecules are confined to the cytoplasm of the host cells. The phage coat fusions are exposed briefly to the cytoplasm during translation but are rapidly secreted through the inner membrane into the

10 periplasmic compartment, remaining anchored in the membrane by their C-terminal hydrophobic domains, with the N-termini, containing the peptides, protruding into the periplasm while awaiting assembly into phage particles. The peptides in the LacI and phage libraries may differ significantly as a result of their exposure to different proteolytic activities. The phage coat proteins require transport across the inner membrane and signal

15 peptidase processing as a prelude to incorporation into phage. Certain peptides exert a deleterious effect on these processes and are underrepresented in the libraries (Gallop et al. (1994) *J. Med. Chem.* 37(9):1233-1251). These particular biases are not a factor in the LacI display system.

The number of small peptides available in recombinant random libraries is enormous.

20 Libraries of 10^7-10^9 independent clones are routinely prepared. Libraries as large as 10^{11} recombinants have been created, but this size approaches the practical limit for clone libraries. This limitation in library size occurs at the step of transforming the DNA containing randomized segments into the host bacterial cells. To circumvent this limitation, an *in vitro* system based on the display of nascent peptides in polysome complexes has

25 recently been developed. This display library method has the potential of producing libraries 3-6 orders of magnitude larger than the currently available phage/phagemid or plasmid libraries. Furthermore, the construction of the libraries, expression of the peptides, and screening, is done in an entirely cell-free format.

In one application of this method (Gallop et al. (1994) *J. Med. Chem.* 37(9):1233-

30 1251), a molecular DNA library encoding 10^{12} decapeptides was constructed and the library

48

expressed in an *E. coli* S30 *in vitro* coupled transcription/translation system. Conditions were chosen to stall the ribosomes on the mRNA, causing the accumulation of a substantial proportion of the RNA in polysomes and yielding complexes containing nascent peptides still linked to their encoding RNA. The polysomes are sufficiently robust to be affinity purified on immobilized receptors in much the same way as the more conventional recombinant peptide display libraries are screened. RNA from the bound complexes is recovered, converted to cDNA, and amplified by PCR to produce a template for the next round of synthesis and screening. The polysome display method can be coupled to the phage display system. Following several rounds of screening, cDNA from the enriched pool of polysomes was cloned into a phagemid vector. This vector serves as both a peptide expression vector, displaying peptides fused to the coat proteins, and as a DNA sequencing vector for peptide identification. By expressing the polysome-derived peptides on phage, one can either continue the affinity selection procedure in this format or assay the peptides on individual clones for binding activity in a phage ELISA, or for binding specificity in a completion phage ELISA (Barret, et al. (1992) *Anal. Biochem* 204,357-364). To identify the sequences of the active peptides one sequences the DNA produced by the phagemid host.

Secondary Screens

The high through-put assays described above can be followed by secondary screens in order to identify further biological activities which will, e.g., allow one skilled in the art to differentiate agonists from antagonists. The type of a secondary screen used will depend on the desired activity that needs to be tested. For example, an assay can be developed in which the ability to inhibit an interaction between a protein of interest and its respective ligand can be used to identify antagonists from a group of peptide fragments isolated though one of the primary screens described above.

Therefore, methods for generating fragments and analogs and testing them for activity are known in the art. Once the core sequence of interest is identified, it is routine to perform for one skilled in the art to obtain analogs and fragments.

This invention is further illustrated by the following examples which in no way should be construed as being further limiting. The contents of all cited references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by

5   reference.

Examples

Generation of a Decorin Construct

pGEMDec containing the 1.7 kb human Decorin cDNA was partially digested with

10   BspH1 and annealed oligonucleotides BSPHXHO1 and BSPHXHO2 (CATGCTCGAGCCGCCAC (SEQ ID NO:1) and CATGGTGGCGGCTCGAG (SEQ ID NO:2), respectively) were ligated to the BspHI site immediately upstream of the human Decorin translation start site. This created an optimal Kozak ribosomal binding sequence as well as an XhoI cloning site. This plasmid was then mutated to introduce an XhoI site 2 bp

15   downstream of the human Decorin translation Stop codon. The 1.1 kb XhoI fragment containing the entire human Decorin coding sequence was then isolated and ligated to goat Beta Casein expression vector BC451 opened with XhoI, creating the BC543 human Decorin mammary expression cassette. This plasmid was fully sequenced to verify orientation and for potential mutations.

20

Preparation of Injection Fragments

The goat Beta Casein - human Decorin expression cassette was separated from the plasmid backbone by digesting to completion with NotI, and prepared for microinjection using the "Wizard" method. Plasmid DNA (100 μg) was separated from the vector backbone

25   by digesting to completion with NotI. The digest was then electrophoresed in an agarose gel, using 1X TAE (Maniatis, T., Fritsch, E. F., and Sambrook, J. 1983. Molecular Cloning, A Laboratory Manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.) as running buffer. The region of the gel containing the DNA fragment corresponding to the expression cassette was visualized under UV light (long wave). The band containing the

30   DNA of interest was excised, transferred to a dialysis bag, and the DNA was isolated by electro-elution in 1X TAE.

Following electro-elution, the DNA fragment was concentrated and cleaned-up by
using the "Wizard DNA clean-up system" (Promega, Cat # A7280), following the provided
protocol and eluting in 125 ml of microinjection buffer (10 mM Tris pH 7.5, EDTA 0.2 mM).
Fragment concentration was evaluated by comparative agarose gel electrophoresis. The
deduced concentrations of the microinjection fragment stock was 150 ng/ml. The stock was
diluted in microinjection buffer just prior to pronuclear injections so that the final
concentration was 1.5 ng/ml.

Microinjection

CD1 female mice were superovulated and fertilized ova were retrieved from the
oviduct. Male pronuclei were then microinjected with DNA diluted in microinjection buffer.
Microinjected embryos were either cultured overnight in CZB media or transferred
immediately into the oviduct of pseudopregnant recipient CD1 female mice. Twenty to thirty
2-cell or forty to fifty one-cell embryos were transferred to each recipient female and allowed
to proceed to term.

Identification of Founder Animals

Genomic DNA was isolated from tail tissue by precipitation with isopropanol and
analyzed by polymerase chain reaction (PCR) for the presence the chicken beta-globin
insulator DNA sequence. For the PCR reactions, approximately 250 ng of genomic DNA
was diluted in 50 µl of PCR buffer (20 mM Tris pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$,
100 µM deoxynucleotide triphosphates, and each primer at a concentration of 600 nM) with
2.5 units of Taq polymerase and processed using the following temperature program:

1 cycle 94°C	60 sec	
5 cycles	94°C	30 sec
	58°C	45 sec
	74°C	45 sec
30 cycles	94°C	30 sec
	55°C	30 sec
	74°C	30 sec

Primer sets:

GBC 332: TGTGCTCCTCTCCATGCTGG (SEQ ID NO:3)

GBC 386: TGGTCTGGGGTGACACATGT (SEQ ID NO:4)

5   Mouse milking:

Female mice were allowed to deliver their pups naturally, and were generally milked

on days 7 and 9 postpartum. Mice were separated from their litters for approximately one

hour prior to the milking procedure. After the one hour holding period, mice were induced to

lactate using an intraperitoneal injection of 5 i. U. Oxytocin in sterile Phosphate Buffered

10   Saline, using a 25 gauge needle. Hormone injections were followed by a one to five minutes

waiting period for the Oxytocin to take effect.

A suction and collection system consisting of a 15 ml conical tube sealed with a

rubber stopper with two 18 gauge needles inserted in it, the hub end of one needle being

inserted into rubber tubing connected to a human breast pump, was used for milking. Mice

15   were placed on a cage top, held only by their tail and otherwise not restricted or confined.

The hub end of the other needle was placed over the mice's teats (one at a time) for the

purpose of collecting the milk into individual eppendorf tube placed in the 15 ml conical

tube. Eppendorf tubes were changed after each sample collection. Milking was continued

until at least 150 μl of milk had been obtained. After collection, mice were returned to their

20   litters.

Protein analysis:

Western Blot analysis was carried out as described in Harlow and Lane, 1988.

Antibodies, A Laboratory Manual. Cold Spring Harbor, New York: Cold Spring Harbor

25   Laboratory, using human Decorin specific Rabbit polyclonal antibody (Chemicon, cat#

AB1909).

Results

30   Transgenic mice:

A total of 805 embryos were microinjected. 624 (77.5 %) embryos survived microinjection, and 537 were transferred to 20 pseudopregnant recipient mice. A total 142 founder mice were born (26.4 % of transferred embryos) and were analyzed by PCR using primers specific for the insulator sequence.

A total of 15 transgenic founders were identified (10.5 %, 1.86 % of microinjected embryo), 6 of which were selected for mating. Human Decorin expression in milk for these lines is summarized in Table 1

Table 1: Expression of human Decorin by transgenic mice carrying the BC543 transgene as determined by western blotting. N/A, not available.

Founder (sex)	PCR positive offspring (only females were analyzed)	human Decorin level in milk (mg/ml) analyzed by Western blot
14 (F)		0.25
22 (F)	227	0.1
36 (F)		1-1.5
62 (F)	215	2-4
73 (M)	152	N/A 5-10
102 (F)	269	0.5 1-1.5

Glycosylation of Decorin expressed in milk:

Decorin is expressed at high level in the milk of transgenic mice carrying the BC543 construct. Four of six lines expressed at levels superior to 1 mg/ml. Of the two lines that expressed at lower levels, one was clearly mosaic (line 14) since no transmission of the transgene to offspring was observed. One surprising aspect of transgenically expressed Decorin is that it migrates on SDS-PAGE as a relatively tight set of bands between

approximately 45 kd to 53 kd, whereas mammalian cell culture derived Decorin migrates as a smear extending approximately from 60 to 120 kd. This migration pattern of the transgenically expressed Decorin is consistent with a molecule that does not contain the glucosaminoglycan chain.

Human Decorin was expressed at high-levels (>1 mg/ml) in the milk of transgenic animals. The transgenically expressed human Decorin does not contain the glycosaminoglycan side chain. This unexpected property of the transgenically expressed human Decorin is very useful since glucosaminoglycan side chains are very heterogeneous and are an obstacle to achieving the necessary uniform production characteristics of therapeutic recombinant protein. Moreover, glucosaminoglycan chains do not appear to be required for the activity of human Decorin as it would be used in a therapeutic context.

Generation and Characterization of Transgenic Goats

A founder (F_0) transgenic goat can be made by transfer of fertilized goat eggs that have been microinjected with a construct (e.g., a BC355 vector containing the human decorin gene operably linked to the regulatory elements of the goat beta-casein gene). The methodologies that follow in this section can be used to generate transgenic goats.

Goat Species and breeds:

Swiss origin goats, e.g., the Alpine, Saanen, and Toggenburg breeds, are useful in the production of transgenic goats.

The sections outlined below briefly describe the steps required in the production of transgenic goats. These steps include superovulation of female goats, mating to fertile males and collection of fertilized embryos. Once collected, pronuclei of one-cell fertilized embryos are microinjected with DNA constructs. All embryos from one donor female are kept together and transferred to a single recipient female if possible.

Goat superovulation:

The timing of estrus in the donors is synchronized on Day 0 by 6 mg subcutaneous norgestomet ear implants (Syncromate-B, CEVA Laboratories, Inc., Overland Park, KS).

54

Prostaglandin is administered after the first seven to nine days to shut down the endogenous synthesis of progesterone. Starting on Day 13 after insertion of the implant, a total of 18 mg of follicle-stimulating hormone (FSH - Schering Corp., Kenilworth, NJ) is given intramuscularly over three days in twice-daily injections. The implant is removed on Day 14.

5   Twenty-four hours following implant removal the donor animals are mated several times to fertile males over a two-day period (Selgrath, et al., Theriogenology, 1990. pp. 1195-1205).

Embryo collection:

Surgery for embryo collection occurs on the second day following breeding (or 72

10   hours following implant removal). Superovulated does are removed from food and water 36 hours prior to surgery. Does are administered 0.8 mg/kg Diazepam

(Valium®), IV, followed immediately by 5.0 mg/kg Ketamine (Keteset), IV. Halothane (2.5%) is administered during surgery in 2 L/min oxygen via an endotracheal tube. The reproductive tract is exteriorized through a midline laparotomy incision. Corpora lutea,

15   unruptured follicles greater than 6 mm in diameter, and ovarian cysts are counted to evaluate superovulation results and to predict the number of embryos that should be collected by oviductal flushing. A cannula is placed in the ostium of the oviduct and held in place with a single temporary ligature of 3.0 Prolene. A 20 gauge needle is placed in the uterus approximately 0.5 cm from the uterotubal junction. Ten to twenty ml of sterile phosphate

20   buffered saline (PBS) is flushed through the cannulated oviduct and collected in a Petri dish. This procedure is repeated on the opposite side and then the reproductive tract is replaced in the abdomen. Before closure, 10-20 ml of a sterile saline glycerol solution is poured into the abdominal cavity to prevent adhesions. The linea alba is closed with simple interrupted sutures of 2.0 Polydioxanone or Supramid and the skin closed with sterile wound clips.

25   Fertilized goat eggs are collected from the PBS oviductal flushings on a stereomicroscope, and are then washed in Ham's F12 medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS) purchased from Sigma. In cases where the pronuclei are visible, the embryos is immediately microinjected. If pronuclei are not visible, the embryos are placed in Ham's F12 containing 10% FBS for short term culture at 37°C in a

30   humidified gas chamber containing 5% CO_2 in air until the pronuclei become visible (Selgrath, et al., Theriogenology, 1990. pp. 1195-1205).

Microinjection procedure:

One-cell goat embryos are placed in a microdrop of medium under oil on a glass depression slide. Fertilized eggs having two visible pronuclei are immobilized on a flame-polished holding micropipet on a Zeiss upright microscope with a fixed stage using Normarski optics. A pronucleus is microinjected with the DNA construct of interest, e.g., a BC355 vector containing the human decorin gene operably linked to the regulatory elements of the goat beta-casein gene, in injection buffer (Tris-EDTA) using a fine glass microneedle (Selgrath, et al., Theriogenology, 1990. pp. 1195-1205).

Embryo development:

After microinjection, the surviving embryos are placed in a culture of Ham's F12 containing 10% FBS and then incubated in a humidified gas chamber containing 5% CO_2 in air at 37°C until the recipient animals are prepared for embryo transfer (Selgrath, et al., Theriogenology, 1990. p. 1195-1205).

Preparation of recipients:

Estrus synchronization in recipient animals is induced by 6 mg norgestomet ear implants (Syncromate-B). On Day 13 after insertion of the implant, the animals are given a single non-superovulatory injection (400 I.U.) of pregnant mares serum gonadotropin (PMSG) obtained from Sigma. Recipient females are mated to vasectomized males to ensure estrus synchrony (Selgrath, et al., Theriogenology, 1990. pp. 1195-1205).

Embryo Transfer:

All embryos from one donor female are kept together and transferred to a single recipient when possible. The surgical procedure is identical to that outlined for embryo collection outlined above, except that the oviduct is not cannulated, and the embryos are transferred in a minimal volume of Ham's F12 containing 10% FBS into the oviductal lumen via the fimbria using a glass micropipet. Animals having more than six to eight ovulation points on the ovary are deemed unsuitable as recipients. Incision closure and post-operative

care are the same as for donor animals (see, e.g., Selgrath, et al., Theriogenology, 1990. pp. 1195-1205).

Monitoring of pregnancy and parturition:

Pregnancy is determined by ultrasonography 45 days after the first day of standing estrus. At Day 110 a second ultrasound exam is conducted to confirm pregnancy and assess fetal stress. At Day 130 the pregnant recipient doe is vaccinated with tetanus toxoid and Clostridium C&D. Selenium and vitamin E (Bo-Se) are given IM and Ivermectin was given SC. The does are moved to a clean stall on Day 145 and allowed to acclimatize to this environment prior to inducing labor on about Day 147. Parturition is induced at Day 147 with 40 mg of PGF2a (Lutalyse®, Upjohn Company, Kalamazoo Michigan). This injection is given IM in two doses, one 20 mg dose followed by a 20 mg dose four hours later. The doe is under periodic observation during the day and evening following the first injection of Lutalyse® on Day 147. Observations are increased to every 30 minutes beginning on the morning of the second day. Parturition occurred between 30 and 40 hours after the first injection. Following delivery the doe is milked to collect the colostrum and passage of the placenta is confirmed.

Verification of the transgenic nature of F_0 animals:

To screen for transgenic F_0 animals, genomic DNA is isolated from two different cell lines to avoid missing any mosaic transgenics. A mosaic animal is defined as any goat that does not have at least one copy of the transgene in every cell. Therefore, an ear tissue sample (mesoderm) and blood sample are taken from a two day old F_0 animal for the isolation of genomic DNA (Lacy, et al., A Laboratory Manual, 1986, Cold Springs Harbor, NY; and Herrmann and Frischauf, Methods Enzymology, 1987. 152: pp. 180-183). The DNA samples are analyzed by the polymerase chain reaction (Gould, et al., Proc. Natl. Acad. Sci, 1989. 86:pp. 1934-1938) using primers specific for human decorin gene and by Southern blot analysis (Thomas, Proc Natl. Acad. Sci., 1980. 77:5201-5205) using a random primed human decorin cDNA probe (Feinberg and Vogelstein, Anal. Bioc., 1983. 132: pp. 6-13).

Assay sensitivity is estimated to be the detection of one copy of the transgene in 10% of the somatic cells.

5

Generation and Selection of production herd

The procedures described above can be used for production of transgenic founder (F_0) goats, as well as other transgenic goats. The transgenic F_0 founder goats, for example, are bred to produce milk, if female, or to produce a transgenic female offspring if it is a male

10 founder. This transgenic founder male, can be bred to non-transgenic females, to produce transgenic female offspring.

Transmission of transgene and pertinent characteristics

Transmission of the transgene of interest, in the goat line is analyzed in ear tissue and

15 blood by PCR and Southern blot analysis. For example, Southern blot analysis of the founder male and the three transgenic offspring shows no rearrangement or change in the copy number between generations. The Southern blots are probed with human decorin cDNA probe. The blots are analyzed on a Betascope 603 and copy number determined by comparison of the transgene to the goat beta casein endogenous gene.

20

Evaluation of expression levels

The expression level of the transgenic protein, in the milk of transgenic animals, is determined using enzymatic assays or Western blots.

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All patents and other references cited herein are hereby incorporated by reference. Other embodiments are within the following claims.